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(54) Title: ANGIOGENIN RECEPTOR, COMPOSITIONS AND METHODS RELATED THERETO

(57) Abstract

The subject invention is directed to a novel isolated angiogenin receptor expressed by endothelial cells, as well as fragments thereof, particularly those which retain angiogenin binding activity. The invention also provides nucleic acids, e.g., which encode the angiogenin receptor, which can be used as probes to detect an angiogenin receptor–encoding nucleic acid, and/or which can be used in antisense methods to inhibit expression of the receptor. The invention also provides antibodies specifically immunoreactive with the subject receptors, as well as cells ectopically expressing the receptor and methods for isolating the receptor or fragment thereof, drug screening assays, and therapeutic and diagnostic methods and compositions related thereto.

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Angiogenin Receptor, Compositions and Methods Related Thereto

Background of the Invention

Field of the Invention

Embodiments of the present invention relate in general to novel cell surface receptors for angiogenin. Embodiments of the present invention also relate to methods for regulating expression of cell surface receptors for angiogenin, and further to methods of stimulating cell proliferation. Embodiments of the present invention also relate to therapeutic methods for treating conditions associated with abnormal angiogenesis.

Description of Related Art

Angiogenin is a potent inducer of angiogenesis (Fett et al., 1985, Biochemistry 24: 5480-5486), a complex process of blood vessel formation that consists of several separate but interconnected steps at the cellular and biochemical level: (i) activation of endothelial cells by the action of an angiogenic stimulus, (ii) invasion of activated endothelial cells into the surrounding tissues and migration toward the source of the angiogenic stimulus, and (iii) proliferation and differentiation of endothelial cells to form a new microvasculature (Folkman and Shing, 1992, J. Biol. Chem. 267: 10931-10934; Moscatelli and Rifkin, 1988, Biochim. Biophys. Acta 948: 67-85). Angiogenin has been demonstrated to induce most of the individual events in the process of angiogenesis including binding to endothelial cells (Badet et al., 1989, Proc. Natl. Acad. Sci. USA 86: 8427-8431), stimulating second messengers (Bicknell and Vallee, 1988, Proc. Natl. Acad. Sci. USA 85: 5961-5965), mediating cell adhesion (Soncin, 1992, Proc. Natl. Acad. Sci. USA 89: 2232-2236), activating cell-associated proteases (Hu and Riordan, 1993, Biochem. Biophys. Res. Commun. 197: 682-687), inducing cell invasion (Hu et al., 1994, Proc. Natl. Acad. Sci. USA 91: 12096-12100) and organizing the formation of tubular structures from cultured endothelial cells (Jimi et al., 1995, Biochem. Biophys. Res. Commun. 211: 476-483). Angiogenin has also been shown to undergo nuclear translocation in endothelial cells via receptor-mediated endocytosis (Moroianu and Riordan, 1994, Proc. Natl. Acad. Sci. USA 91: 1677-1681) and nuclear localization sequence-assisted nuclear import (Moroianu and Riordan, 1994, Biochem. Biophys. Res. Commun. 203: 1765-1772).

A unique feature of angiogenin is that although it was originally isolated from the conditioned medium of human adenocarcinoma cells (Fett et al., 1985, supra) based solely on its angiogenic activity on the chick chorioallantoic membrane (CAM), it actually is a constituent of human plasma and normally circulates at a concentration of 250 to 360 ng/ml (Shimoyama et al., 1996, Cancer Res. 56: 2703-2706; Bläser et al., 1993, Eur. J. Clin. Chem. Clin. Biochem. 31: 513-516). Angiogenesis is a highly controlled process under usual physiological conditions. Abnormal angiogenesis can have devastating consequences as in many pathological conditions such as arthritis, diabetic retinopathy and tumor growth.

In order to avoid unwanted, rampant angiogenesis, circulating angiogenin must be poised for action without actively stimulating neovascularization. On the other hand, extravascular angiogenin does stimulate the formation of blood vessels as evidenced by the angiogenesis seen in the chick embryo CAM assay (Fett et al., 1985, supra), in the cornea and meniscus of the knee of the rabbit (King and Vallee, 1991, J. Bone Joint Surg. 73-B: 587-590) and by the ability of angiogenin antagonists to inhibit the establishment of cell tumors implanted in nude mice (Olson et al., 1995, Proc. Natl. Acad. Sci. USA 92: 442-446).

The mechanism by which angiogenin stimulates angiogenesis has not been elucidated mainly due to lack of evidence that it is mitogenic and the inability to identify its cellular receptor protein. A conceivable mechanism to regulate the activity of angiogenin would act at the cellular level and control the expression and manifestation of its receptors. We have previously identified an angiogenin binding protein located on the endothelial cell surface (Hu et al., 1991, Proc. Natl. Acad. Sci. USA 88: 2227-2231), characterized it as a dissociable α-smooth muscle type actin (Hu et al., 1991, supra), and showed that it is involved in angiogenin-induced angiogenesis by stimulating cell-associated proteolytic activities and endothelial cell invasion (Hu et al., 1994, supra). However, a classical transmembrane cellular receptor protein for angiogenin has remained elusive.

The mitogenic activity of angiogenin toward endothelial cells has been another open question. Most known angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), and epidermal growth factor are all endothelial cell mitogens (Folkman and Klagsbrun, 1987, Science 235: 442-447). But the mitogenic activity of angiogenin has not been established except for an early study showing that bovine angiogenin stimulates ^{3}H-thymidine uptake and proliferation of bovine brain capillary endothelial cells, but not of

bovine aortic arch endothelial cells (Chamoux et al., 1991, Biochem. Biophys. Res. Commun. 176: 833-839). It has been reported that initial vascular sprouting and elongation can be achieved merely by migration and redistribution of existing endothelial cells from the limbal vessels and does not require proliferation of endothelial cells (Sholley et al., 1984, Lab. Invest. 51: 624-634). However, subsequent vascular ingrowth will not progress without cell proliferation.

Accordingly, there is a need to identify cell surface receptors for angiogenin and the mechanisms by which angiogenin stimulates angiogenesis. There is a further need to develop angiogenin cell surface receptors which can be used in methods to therapeutically treat conditions associated with normal angiogenesis, such as those occurring in pathological conditions such as arthritis, diabetic retinopathy and tumor growth, and in physiological processes such as wound repair, reproduction and development.

Summary of the Invention

Embodiments of the present invention are directed to a novel isolated angiogenin cellular receptor protein ("AR") expressed on the membrane surface of endothelial cells, as well as fragments thereof, particularly those which retain angiogenin binding activity. The invention also provides nucleic acids, e.g., which encode the angiogenin receptor, which can be used as probes to detect an AR-encoding nucleic acid, and/or which can be used in antisense methods to inhibit expression of the receptor. The invention also provides antibodies specifically immunoreactive with the subject receptors, as well as cells ectopically expressing the receptor and methods for isolating the receptor or fragments thereof.

Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, and inhibition of angiogenesis. For instance, embodiments of the present invention are directed to methods for stimulating the growth of human endothelial cells using angiogenin under certain reaction conditions. Other embodiments of the present invention are further directed to methods for regulating expression of the cellular receptor protein under certain reaction conditions. An alternate embodiment of the present invention is directed to methods of therapeutically treating conditions associated with abnormal angiogenesis.

According to the present invention, human endothelial cells in low concentration are contacted with a growth stimulating amount of angiogenin to stimulate the growth and proliferation of endothelial cells. In addition, a 170 kDa cellular receptor protein for

angiogenin which is expressed only at low endothelial cell concentration has been discovered and isolated. This angiogenin receptor is useful, for example, as a therapeutic agent and as a component in a diagnostic assay for angiogenin. Polyclonal or monoclonal antibodies against the angiogenin receptor are raised by methods well known in the art and are useful in methods for inhibiting the angiogenin receptor.

According to an alternate embodiment of the present invention, a method for therapeutically treating abnormal angiogenesis is disclosed wherein an isolated 170 kDa cellular receptor protein for angiogenin is used to inhibit angiogenesis by binding to angiogenin. According to a further alternate embodiment of the present invention, a method for inhibiting angiogenesis is disclosed wherein fragments and derivatives of the 170 kDa cellular receptor protein for angiogenin are used to treat angiogenesis-based diseases. According to a further alternate embodiment of the present invention, a method for therapeutically treating a condition associated with angiogenesis is disclosed wherein the 170 kDa cellular receptor protein for angiogenin is inhibited in a manner to reduce angiogenesis. According to a further alternate embodiment of the present invention, a method for promoting angiogenesis is disclosed wherein the 170 kDa cellular receptor protein for angiogenin is activated to enhance processes such as wound healing and repair.

Brief Description of Drawings

Fig. 1 is a graph depicting the results of an experiment demonstrating dose-dependent stimulation of ^{3}H-thymidine incorporation and proliferation of endothelial cells by angiogenin.

Fig. 2 is an autoradiograph showing binding and crosslinking of angiogenin to a 170 kDa protein.

Fig. 3 is a graph showing concentration dependent binding of angiogenin to HUVE cells and Scatchard analysis of the binding data.

Fig. 4 is an SDS-PAGE analysis of the 170 kDa angiogenin receptor. Lanes A and B were the samples from a deactivated Sepharose and an angiogenin-Sepharose affinity column, respectively.

Detailed Description of Certain Preferred Embodiments

Angiogenesis, or the proliferation of new capillary blood vessels, is a fundamental process necessary for normal growth and development of tissues. It is a prerequisite for the development and differentiation of the vascular tree, as well as for a wide variety of fundamental physiological processes including embryogenesis, somatic growth, tissue and organ repair and regeneration, cyclical growth of the corpus luteum and endometrium, and development and differentiation of the nervous system. In the female reproductive system, angiogenesis occurs in the follicle during its development, in the corpus luteum following ovulation and in the placenta to establish and maintain pregnancy. Angiogenesis additionally occurs as part of the body's repair processes, e.g. in the healing of wounds and fractures.

Abnormal angiogenesis (that is to say, stimulation of the growth of new blood vessels owing to a pathological syndrome) is an established characteristic of many diseases, especially diabetic retinopathy, rheumatoid arthritis, haemangioma and the growth of solid tumours. For instance, angiogenesis is a factor in tumor growth since a tumor must continuously stimulate growth of new capillary blood vessels in order to grow. Angiogenesis can also play an important part in other diseases, such as arterio-coronary disease.

The regulation of angiogenesis is an important mechanism for developmental control in both normal and disease states. The present invention concerns the discovery of a new family of angiogenin receptors, also referred to herein as "AR proteins" or "ARs", which are demonstrated to bind to angiogenin polypeptides with high affinity. As described herein, the subject receptors exhibit expression domains indicative of an important role in angiogenin-mediated signalling.

Accordingly, certain aspects of the present invention relate to nucleic acids encoding AR polypeptides, the AR polypeptides themselves (including various fragments), antibodies immunoreactive with AR proteins, and preparations of such compositions. Moreover, the present invention provides diagnostic and therapeutic assays and reagents for detecting and treating disorders involving, for example, aberrant expression (or loss thereof) of AR, AR ligands (particularly angiogenin proteins), or signal transducers thereof.

In addition, drug discovery assays are provided for identifying agents which can modulate the biological function of AR proteins, such as by altering the binding of the receptor to angiogenin proteins or other extracellular/matrix factors, or the ability of the bound AR protein to transduce angiogenin signals. Such agents can be useful

therapeutically to alter the growth, maintenance and/or differentiation of a tissue, particularly endothelially-derived tissue. Other aspects of the invention are described below or will be apparent to those skilled in the art in light of the present disclosure.

For convenience, certain terms employed in the specification and appended claims are collected here.

The term "angiogenin receptor" or "AR" refers to a family of polypeptides characterized at least in part by being identical or sharing a degree of sequence homology with all or a portion of the receptor polypeptide described in the appended examples, e.g., containing amino acid sequence(s) identical or homologous with the peptidyl sequences represented in SEQ ID No: 1, 2, 3 or 5. The subject receptor polypeptides can be cloned or purified from any of a number of eukaryotic organisms, especially vertebrates, and particularly mammals. Moreover, other AR polypeptides can be generated according to the present invention, which polypeptides do not ordinarily exist in nature but rather are generated by non-natural mutagenic techniques.

A number of features of the human angiogenin receptor have been observed upon inspection. In particular, it is noted that at least one form of the receptor includes an extracellular domain, e.g., and therefore apparently has a secretion signal sequence (e.g., a peptidyl portion which causes extracellular secretion of at least a portion of the protein). The protein also has an apparent membrane-anchoring domain, e.g., presumably in the form of a transmembrane domain, though other possible membrane-anchoring domains may be incorporated in the protein, e.g., such as RGD sequences, GPI-linkages and the like. The receptor is also characterized by its tight binding of angiogenin, having an apparent dissociation constant in the nanomolar range. Additionally, comparison of the partial sequence data reveals homology of the angiogenin receptor with certain receptor tyrosine kinases (RTKs), suggesting that the subject angiogenin receptor may also be an RTK.

A "membrane-anchoring" region refers to a sequence of amino acids that is capable of retaining the AR polypeptide at the cell surface.

A "glycosylated" AR polypeptide has one or more covalent linkages with α-glycosyl groups (e.g. derivatized with a carbohydrate). For instance, the AR protein can be glycosylated on an existing residue, or can be mutated to preclude carbohydrate attachment, or can be mutated to provide new glycosylation sites, such as for N-linked or O-linked glycosylation.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding an AR polypeptide, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding an AR polypeptide and comprising AR-encoding exon sequences, though it may optionally include intron sequences which are derived from, for example, a chromosomal angiogenin receptor gene or from an unrelated chromosomal gene. The term "intron" refers to a DNA sequence present in a given AR gene which is not translated into protein and is generally found between exons.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of an AR polypeptide or, where anti-sense expression occurs from the transferred gene, the expression of a naturally-occurring form of the AR protein is disrupted.

As used herein, the term "specifically hybridizes" refers to the ability of a nucleic acid probe/primer of the invention to hybridize to at least 15, 25, 50 or 100 consecutive nucleotides of an AR gene, or a sequence complementary thereto, or naturally occurring mutants thereof, such that it has less than 15%, preferably less than 10%, and more preferably less than 5% background hybridization to a cellular nucleic acid (e.g., mRNA or genomic DNA) encoding a protein other than an AR protein, as defined herein.

As used herein, "phenotype" refers to the entire physical, biochemical, and physiological makeup of a cell, e.g., having any one trait or any group of traits.

The terms "induction" or "induce", as relating to the biological activity of an angiogenin protein, refer generally to the process or act of causing to occur a specific effect on the phenotype of a cell. Such effects can be in the form of causing a change in the phenotype, e.g., differentiation to another cell phenotype, or can be in the form of maintaining the cell in a particular phenotype, e.g., preventing dedifferentation or promoting survival of a cell.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and expressing coding sequences to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of a recombinant AR gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of AR genes.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In an exemplary transgenic animal, the transgene causes cells to express a recombinant form of an angiogenin receptor, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant AR gene is silent are also

contemplated, as for example, the FLP or CRE recombinase dependent constructs. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more AR genes is caused by human intervention, including both recombination and antisense techniques.

The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, livestock, avian species, amphibians, reptiles, etc. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that a recombinant AR gene is present and/or expressed or disrupted in some tissues but not others.

As is well known, genes for a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding an AR polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individuals of the same species, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a protein with the same biological activity.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an AR sequence of the present invention.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur

in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding an AR polypeptide with a second amino acid sequence defining a domain (e.g. polypeptide portion) foreign to and not substantially homologous with any domain of an AR protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula X-AR-Y, wherein AR represents a portion of the fusion protein which is derived from an AR protein, and X and Y are, independently, absent or represent amino acid sequences which are not related to AR sequences in an organism.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to a transcriptional regulatory sequence(s). Transcription of the reporter gene is controlled by such sequences. The activity of at least one or more of these control sequences is directly or indirectly regulated by a signal transduction pathway involving a phospholipase, e.g., is directly or indirectly regulated by a second messenger produced by the phospholipase activity. The transcriptional regulatory sequences can include a promoter and other regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or regulatory sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or regulatory sequences that are recognized by effector molecules, including those that are specifically induced upon activation of a phospholipase. For example, modulation of the activity of the promoter may be effected by altering the RNA polymerase binding to the promoter region, or, alternatively, by interfering with initiation of transcription or elongation of the mRNA. Such sequences are herein collectively referred to as transcriptional regulatory elements or sequences. In addition, the construct may include sequences of nucleotides that alter the stability or rate of translation of the resulting mRNA in response to second messages, thereby altering the amount of reporter gene product.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding an AR polypeptide preferably includes no more than 10 kilobases (kb) of

nucleic acid sequence which 4naturally immediately flanks the AR gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals only undergo angiogenesis in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The control of angiogenesis is a highly regulated system of angiogenic stimulators and inhibitors. The control of angiogenesis has been found to be altered in certain disease states and, in many cases, the pathological damage associated with the disease is related to the uncontrolled angiogenesis.

A "patient" or "subject" to be treated can mean either a human or non-human animal.

In one aspect, the present invention features a purified or recombinant AR polypeptide having a core peptide chain with a molecular weight of about 170kDa. In other embodiments, the AR polypeptide may preferably have a molecular weight of about 55kDa, and include an amino acid sequence similar or identical to SEQ ID No. 1, 2, 3 and/or 5. As described in the appended examples, the subject angiogenin receptor shares certain sequence homologies with the RTK protein Ros 1. It is therefore possible that certain full-length forms of the subject angiogenin receptor protein will be about the same size as the Ros 1 protein, e.g., having a molecular weight of about 265kDa (e.g., +/- 10 kd). It will also be understood that certain post-translational modifications, e.g., glycosylation, phosphorylation and the like, can increase the apparent molecular weight of the AR protein relative to the unmodified polypeptide chain, and cleavage of certain sequences, such as pro-sequences, can likewise decrease the apparent molecular weight. Other preferred AR polypeptides include: a mature AR polypeptide which lacks a signal sequence peptide; a mature, extracellular fragment (soluble) of the receptor. In a preferred embodiment, the AR polypeptide includes an angiogenin binding domain.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of an AR polypeptide which function in a limited capacity as one of either an agonist (e.g., mimics or potentiates a bioactivity of the wild-type AR protein) or an antagonist (e.g., inhibits a bioactivity of the wild-type AR protein), in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function. For example, truncated forms of the angiogenin receptor, e.g., soluble fragments of the extracellular domain, can be provided to competitively inhibit ligand (angiogenin) binding to the wild-type receptor.

Homologs of the subject AR protein can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the AR polypeptide from which it was derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to angiogenin proteins and competing with wild-type AR, or binding to other angiogenin receptors (such as subunits of an angiogenin receptor) to form unresponsive angiogenin receptor complexes. Thus, the AR protein and homologs thereof provided by the subject invention may be either positive or negative regulators of cell growth, death and/or differentiation.

In general, polypeptides referred to herein as having an activity of an angiogenin receptor (e.g., are "bioactive") are defined as polypeptides which include an amino acid sequence corresponding (e.g., identical or homologous) to all or a portion of the amino acid sequences of the AR protein isolated according to the appended examples, e.g., having a polypeptide including one or both amino acid sequences represented in SEQ ID No: 1, 2, 3 or 5, and which agonize or antagonize all or a portion of the biological/biochemical activities of a naturally occurring receptor. Examples of such biological activity include the ability to bind with high affinity angiogenin proteins. The bioactivity of certain embodiments of the subject AR polypeptides can be characterized in terms of an ability to modulate proliferation (and optionally migration) of endothelial cells, e.g, in an angiogenin-dependent fashion, and/or a (tyrosine) kinase activity.

Other biological activities of the subject AR proteins are described herein or will be reasonably apparent to those skilled in the art. According to the present invention, a polypeptide has biological activity if it is a specific agonist or antagonist of a naturally-occurring form of an angiogenin receptor.

The term "recombinant AR polypeptide" refers to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding an AR polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant AR gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native AR protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The present invention also provides methods of producing the subject AR polypeptides. In one embodiment, the angiogenin receptor can be purified from a natural source, such as by the protocols set forth in the appended examples. In other embodiments, the protein is produced by recombinant techniques and purifed by methods suitable for the host cell and/or the presence of additional amino acids in the recombinant protein. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. If the recombinant protein is not provided with a secretion signal peptide, such as in the case of a GST fusion protein, the cells may be harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant AR polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptides. In a preferred embodiment, the recombinant AR polypeptide is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein or poly(His) fusion protein.

This invention also pertains to a host cell transfected to express recombinant forms of the subject AR polypeptides. The host cell may be any eukaryotic or prokaryotic cell. Thus, a nucleotide sequence derived from the cloning of AR proteins, encoding all or a selected portion of a full-length protein, can be used to produce a recombinant form of an AR polypeptide via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g. angiogenin proteins, TGFβ proteins, as well as a wide

range of receptors. Similar procedures, or modifications thereof, can be employed to prepare recombinant AR polypeptides by microbial means or tissue-culture technology in accord with the subject invention.

When it is desirable to express only a portion of an AR protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from E. coli (Ben-Bassat et al. (1987) J. Bacteriol. 169:751-757) and Salmonella typhimurium and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) PNAS 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing AR-derived polypeptides in a host which produces MAP (e.g., E. coli or CM89 or S. cerevisiae), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., supra).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable to produce an immunogenic fragment of an AR protein. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of the AR polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of a subject AR protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising AR epitopes as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of an AR protein and the poliovirus capsid protein can be created to enhance immunogenicity of the set of polypeptide antigens (see, for example, EP Publication No: 0259149; and Evans et al. (1989) Nature 339:385; Huang et al. (1988) J. Virol. 62:3855; and Schlienger et al. (1992) J. Virol. 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can also be utilized to generate an immunogen, wherein a desired portion of an AR polypeptide is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric

branching lysine core (see, for example, Posnett et al. (1988) JBC 263:1719 and Nardelli et al. (1992) J. Immunol. 148:914). Antigenic determinants of AR proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the AR polypeptides of the present invention, particularly truncated forms of the AR protein. For example, AR polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the AR polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)).

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can allow purification of the expressed fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified protein (e.g., see Hochuli et al. (1987) J. Chromatography 411:177; and Janknecht et al. PNAS 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992).

The AR polypeptides may also be chemically modified to create AR derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, cholesterol, phosphate, acetyl groups and the like. Covalent derivatives of AR proteins can be prepared by linking the chemical moieties to functional

groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

As appropriate, formulations of multimeric AR polypeptides are also provided. The multimers of the soluble forms of the subject AR polypeptides may be produced according to the methods known in the art. In one embodiment, the AR multimers are cross-linked chemically by using known methods which will result in the formation of either dimers or higher multimers of the soluble forms of the AR polypeptides. Another way of producing the multimers of the soluble forms of the AR polypeptides is by recombinant techniques, e.g., by inclusion of hinge regions. This linker can facilitate enhanced flexibility of the chimeric protein allowing the various AR monomeric subunits to freely and (optionally) simultaneously interact with an AR ligand by reducing steric hindrance between the two fragments, as well as allowing appropriate folding of each portion to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence $(Gly_4Ser)_3$ can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent Nos. 5,091,513 and 5,258,498. Naturally occurring unstructured linkers of human origin are preferred as they reduce the risk of immunogenicity.

The present invention also makes available isolated AR polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially receptors and/or other inductive polypeptides which may normally be associated with the AR polypeptide. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of AR polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a peptide or DNA or RNA sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits

as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions. In preferred embodiments, purified AR preparations will lack any contaminating proteins from the same animal from which AR is normally produced, as can be accomplished by recombinant expression of, for example, a mammalian AR protein in a yeast or bacterial cell.

Isolated peptidyl portions of AR proteins can also be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, an AR polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") AR protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant AR polypeptides of the present invention also include homologs of the authentic AR proteins, such as versions of those proteins which are resistant to proteolytic cleavage, as for example, due to mutations which alter ubiquitination, prenylation or the like, enzymatic release of the extracellular domain, or other enzymatic targeting associated with the protein.

Modification of the structure of the subject AR polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or post-translational modifications. Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, or to produce specific antagonists thereof, are considered functional equivalents of the AR polypeptides (though they may be agonistic or antagonistic of the bioactivities of the authentic protein). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, Biochemistry, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional AR homolog (e.g. functional in the sense that the resulting polypeptide mimics or antagonizes the authentic form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial point mutants of the subject AR proteins as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in modulating signal transduction and/or ligand binding. The purpose of screening such combinatorial libraries is to generate, for example, novel AR homologs which can act as either agonists or antagonists, or alternatively, possess novel activities all together. To illustrate, AR homologs can be engineered by the present method to provide selective, constitutive activation of angiogenin activity, or alternatively, to be dominant negative inhibitors of AR-dependent signal transduction. For instance, mutagenesis can provide AR homologs which are able to bind extracellular ligands yet are unable to bind or signal through intracellular regulatory proteins. Exemplary techniques which have been employed in the directed evolution of other proteins include Scott et al. (1990) Science

249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; and methods described in U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815.

In an exemplary embodiment, a library of coding sequence fragments can be provided for an AR clone in order to generate a variegated population of AR fragments for screening and subsequent selection of bioactive fragments. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of an AR coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of AR homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity, such as angiogenin binding, facilitates relatively easy isolation of the vector encoding the gene whose product was detected.

The invention also provides for reduction of the receptor to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt a biological activity of the native receptor, e.g. as inhibitors of protein-protein interactions, such as with a ligand of the receptor. Thus, such mutagenic techniques as described above are also useful to map the determinants of the AR proteins which participate in protein-protein interactions involved in, for example, interaction of the subject receptor with angiogenin polypeptides. Alternatively, a similar system can be used to derive fragments of an angiogenin protein which bind to an AR protein and competitively inhibit binding of the full length angiogenin protein.

To further illustrate, the critical residues of either an angiogenin receptor or angiogenin which are involved in molecular recognition of the other can be determined and used to generate AR-derived or angiogenin-derived peptidomimetics which competitively inhibit receptor/ligand interactions. By employing, for example, scanning mutagenesis to map the amino acid residues of a protein which is involved in binding other proteins, peptidomimetic compounds can be generated which mimic those residues which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of an AR protein (or its ligand). For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc* Perkin Trans 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Another aspect of the invention pertains to isolated nucleic acids comprising nucleotide sequences encoding angiogenin receptor polypeptides, and/or equivalents of such nucleic acids. The term nucleic acid as used herein is intended to include fragments as equivalents. The term equivalent is understood to include nucleotide sequences encoding functionally equivalent AR polypeptides or functionally equivalent peptides having an activity of an AR protein such as described herein. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants. Equivalents will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to a human gene encoding an angiogenin receptor, e.g., hybridizes to at least the coding sequence of SEQ ID No: 4 or 6 or the AR sequence of ATCC deposit _____, and preferably to a human gene encoding an angiogenin receptor including amino acid sequences represented in SEQ ID No: 1, 2, 3 or 5.

Preferred nucleic acids encode an AR polypeptide comprising an amino acid sequence at least 60%, 70% or 80% homologous, more preferably at least 85% homologous and most preferably at least 95% homologous with an amino acid sequence of a naturally occurring AR protein, e.g., such as a human angiogenin receptor described in the appended examples, e.g., including one or both of the amino acid sequences represented in SEQ ID No: 1, 2, 3 or 5. Nucleic acids which encode polypeptides at least about 98-99% homology with such sequences are of course also within the scope of the invention, as are nucleic acids identical in sequence with the receptor described in the appended examples and, e.g., having the amino acid sequences enumerated in Sequence listing.

Nucleic acids having a sequence that differs from the AR gene of HUVE cells, e.g., due to degeneracy in the genetic code, are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of an AR polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of an AR polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject AR polypeptides will exist among, for example, humans. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of an AR polypeptide may exist among individuals of a given species due to natural allelic variation.

As used herein, an AR gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence encoding the entire mature form of an AR protein yet which (preferably) encodes a polypeptide which retains some biological activity of the full length protein. Fragment sizes contemplated by the present invention include, for example, 5, 10, 25, 50, 75, 100, or 200 amino acids in length. In a preferred embodiment of a truncated receptor, the polypeptide will include all or a sufficient portion of the ligand domain to bind to an angiogenin polypeptide.

Angiogenin receptor-encoding nucleic acids can be obtained from mRNA present in cells of metazoan organisms, especially vertebrates, and most preferably mammals. It will also be possible to obtain nucleic acids encoding AR polypeptides of the present invention from genomic DNA from both adults and embryos. For example, a gene

encoding an AR protein can be cloned from either a cDNA or a genomic library in accordance with protocols generally known to persons skilled in the art. For instance, a cDNA encoding an AR protein can be obtained by isolating total mRNA from a cell, such as a mammalian cell, e.g. a human cell, as desired, more particularly a human endothelial cell, and probing the nucleic acid with (degenerate) probes/primers based on the microsequencing data set forth in the appended examples. Receptor genes can also be isolated by expression cloning techniques, e.g., using antibodies raised against the purified receptor protein, or by ligand blotting techniques with labeled angiogenin, as described in the appended examples.

Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding a subject AR protein so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes an AR protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of an AR gene. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphorothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775), or peptide nucleic acids (PNAs). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized

in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of routes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolve ' or suspended immediately prior to use. Lyophilized forms are also included.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind. Such diagnostic tests are described in further detail below.

Likewise, the antisense constructs of the present invention, by antagonizing the normal biological activity of an AR protein, e.g., by reducing the level of its expression, can be used in the manipulation of tissue, e.g. tissue maintenance, differentiation or growth, both *in vivo* and *ex vivo*.

Furthermore, the anti-sense techniques (e.g. microinjection of antisense molecules, or transfection with plasmids whose transcripts are anti-sense with regard to an AR mRNA or gene sequence) can be used to investigate the role of the subject receptor in developmental events, as well as the normal cellular function of the receptor in adult tissue. Such techniques can be utilized in cell culture, but can also be used in the creation of transgenic animals (described *infra*).

This invention also provides expression vectors containing a nucleic acid encoding an AR polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject AR proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when

operatively linked to it, may be used in these vectors to express DNA sequences encoding AR polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids, e.g., encoding either an agonistic or antagonistic form of a subject AR protein or an antisense molecule described above. Thus, another aspect of the invention features expression vectors for *in vivo* or *in vitro* transfection and expression of an AR polypeptide or antisense molecule in particular cell types so as to reconstitute the function of, or alternatively, abolishes all or a portion of the biological function of AR-induced transcription in a tissue in which the naturally-occurring form of the protein is misexpressed (or has been disrupted); or to deliver a form of the protein which alters maintenance or differentiation of tissue, or which inhibits neoplastic or hyperplastic proliferation.

Expression constructs of the subject AR polypeptides, as well as antisense constructs, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or calcium phosphate precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such

factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of AR expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA encoding the particular AR polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid. Retrovirus vectors, adenovirus vectors and adeno-associated virus vectors are exemplary recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a subject AR polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject AR polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

Another aspect of the invention pertains to an antibody specifically reactive with an angiogenin receptor protein. For example, by using immunogens derived from an AR protein, e.g. derived from a purified protein or based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., an AR polypeptide or an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an AR protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other

immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization of an animal with an antigenic preparation of a receptor polypeptide, anti-AR antisera can be obtained and, if desired, polyclonal anti-AR antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an AR polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with an AR polypeptide. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab)_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab)_2$ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having affinity for an AR protein conferred by at least one CDR region of the antibody.

Both monoclonal and polyclonal antibodies (Ab) directed against authentic AR polypeptides, or AR variants, and antibody fragments such as Fab, $F(ab)_2$, Fv and scFv can be used to block the action of an AR protein and allow the study of the role of these proteins in, for example, differentiation of tissue. Experiments of this nature can aid in deciphering the role of AR proteins that may be involved in control of proliferation versus differentiation, e.g., in patterning and tissue formation.

Antibodies which specifically bind AR epitopes can also be used in immunohistochemical staining of tissue samples in order to evaluate the abundance and pattern of expression of each of the subject AR polypeptides. Anti-AR antibodies can be used diagnostically in immuno-precipitation and immuno-blotting to detect and evaluate

AR protein levels in tissue as part of a clinical testing procedure. For instance, such measurements can be useful in predictive valuations of the onset or progression of proliferative disorders marked by upregulation of angiogenesis. Likewise, the ability to monitor AR protein levels in an individual can allow determination of the efficacy of a given treatment regimen for an individual afflicted with such a disorder. The level of AR polypeptides may be measured from cells in bodily fluid, such as in samples of cerebral spinal fluid or amniotic fluid, or can be measured in tissue, such as produced by biopsy. Diagnostic assays using anti-AR antibodies can include, for example, immunoassays designed to aid in early diagnosis of a disorder, particularly ones which are manifest at birth. Diagnostic assays using anti-AR polypeptide antibodies can also include immunoassays designed to aid in early diagnosis and phenotyping neoplastic or hyperplastic disorders.

Another application of anti-AR antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as λ gt11, λgt18-23, λZAP, and λORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce fusion proteins. For instance, λgt11 will produce fusion proteins whose amino termini consist of ß-galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of an AR protein, e.g. orthologs of the AR protein from other species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with anti-AR antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of AR homologs can be detected and cloned from other animals, as can alternate isoforms (including splicing variants) from humans.

Moreover, the nucleotide sequences determined from the cloning of AR genes will further allow for the generation of probes and primers designed for use in identifying and/or cloning AR homologs in other cell types, e.g. from other tissues, as well as AR homologs from other organisms.

Such probes can also be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress an AR protein, such as by measuring a level of an AR-encoding nucleic acid in a sample of cells from a patient-animal; e.g. detecting AR mRNA levels or determining whether a genomic AR gene has been mutated or deleted.

To illustrate, nucleotide probes can be generated from the subject AR genes which facilitate histological screening of intact tissue and tissue samples for the presence (or

absence) of receptor-encoding transcripts. Similar to the diagnostic uses of anti-AR antibodies, the use of probes directed to AR messages, or to genomic AR sequences, can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, degenerative disorders marked by loss of particular cell-types, apoptosis, neoplastic and/or hyperplastic disorders (e.g. unwanted cell growth) or abnormal differentiation of tissue. Used in conjunction with immunoassays as described above, the oligonucleotide probes can help facilitate the determination of the molecular basis for a developmental disorder which may involve some abnormality associated with expression (or lack thereof) of an AR protein. For instance, variation in polypeptide synthesis can be differentiated from a mutation in a coding sequence.

Accordingly, the present method provides a method for determining if a subject is at risk for a disorder characterized by aberrant expression of an angiogenin receptor. In preferred embodiments, the method can be generally characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by at least one of (i) an alteration affecting the integrity of a gene encoding an AR-protein, or (ii) the mis-expression of the AR gene. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from an AR gene, (ii) an addition of one or more nucleotides to an AR gene, (iii) a substitution of one or more nucleotides of an AR gene, (iv) a gross chromosomal rearrangement of an AR gene, (v) a gross alteration in the level of a messenger RNA transcript of an AR gene, (vii) aberrant modification of an AR gene, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an AR gene, (viii) a non-wild type level of an AR-protein, and (ix) inappropriate post-translational modification of an AR-protein. As set out below, the present invention provides a large number of assay techniques for detecting lesions in an AR gene, and importantly, provides the ability to discern between different molecular causes underlying AR-dependent aberrant cell growth, proliferation and/or differentiation.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a (purified) oligonucleotide probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of an AR gene, such as encoding an amino acid sequence represented by either of SEQ ID No: 1, 2, 3 or 5, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject AR genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is exposed to

nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels.

In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1944) PNAS 91:360-364), the latter of which can be particularly useful for detecting point mutations in the AR gene. In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to an AR gene under conditions such that hybridization and amplification of the AR gene (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In still another embodiment, the level of an AR protein can be detected by immunoassay. For instance, the cells of a biopsy sample can be lysed, and the level of an AR protein present in the cell can be quantitated by standard immunoassay techniques. In yet another exemplary embodiment, aberrant methylation patterns of an AR gene can be detected by digesting genomic DNA from a patient sample with one or more restriction endonucleases that are sensitive to methylation and for which recognition sites exist in the AR gene (including in the flanking and intronic sequences). See, for example, Buiting et al. (1994) Human Mol Genet 3:893-895. Digested DNA is separated by gel electrophoresis, and hybridized with probes derived from, for example, genomic or cDNA sequences. The methylation status of the AR gene can be determined by comparison of the restriction pattern generated from the sample DNA with that for a standard of known methylation.

In still other embodiments, the ligand binding domain of the AR protein can be used to quantitatively detect the level of AR ligands, e.g., angiogenin proteins. To illustrate, a soluble form of the AR protein can be generated which retains angiogenin binding activity. Samples of bodily fluid(s), e.g., plasma, serum, lymph, marrow, cerebral/spinal fluid, milk, urine and the like can be contacted with the receptor under conditions wherein ligand/receptor binding can occur, and the level of ligand/receptor

complexes formed can be detected by any of a variety of techniques known in the art. For example, competitive binding assays using standardized samples of angiogenin proteins can be used to quantitate the amount of analyte bound from the fluid sample.

In yet other embodiments, such AR protein can be used to detect the presence of an AR ligand on a cell surface. For instance, the AR protein can be contacted with cells from a biopsy, and the ability of the AR protein to decorate (label) certain cells of the sample is ascertained. The binding of the AR protein to cell populations of the sample can be detected, for example, by the use of antibodies against the AR protein, or by detection of a label associated with the AR protein. In the case of the latter, the AR protein can be labeled, for example, by chemical modification or as a fusion protein. Exemplary labels include radioisotopes, fluorescent compounds, enzyme co-factors, which can be added by chemical modification of the protein, and epitope tags such as myc, pFLAG and the like, or enzymatic activities such as GST or alkaline phosphatase which can be added either by chemical modification or by generation of a fusion protein.

Furthermore, the present invention also contemplates the detection of soluble forms of the AR protein in bodily fluid samples. As described in the art, e.g., see Diez-Ruiz et al. (1995) *Eur J Haematol* 54:1-8 and Owen-Schaub et al. (1995) *Cancer Lett* 94:1-8, [describing CNTF receptors] in certain instances soluble forms of receptors are believed to play a role as modulators of the biological function of their cognate ligands in an agonist/antagonist pattern. In various pathologic states, the production and release of soluble AR proteins may mediate host response and determine the course and outcome of disease by interacting with AR ligands such as angiogenin and competing with cell surface receptors. The determination of soluble AR proteins in body fluids is a tool to gain information about various disease states, and may be of prognostic value to a clinician. For example, the level of soluble AR protein in a body fluid may give useful information for monitoring, *inter alia*, neoplastic or hyperplastic transformations.

The level of soluble receptor present in a given sample can be quantitated, in light of the present disclosure, using known procedures and techniques. For example, antibodies immunoselective for the ligand binding domain of the AR protein can be used to detect and quantify its presence in a sample, e.g., by well-known immunoassay techniques. Alternatively, a labeled ligand of the receptor can be used to detect the presence of the receptor in the fluid sample.

A number of techniques exist in the art for now identifying additional ligands to the AR protein. For instance, expression cloning can be carried out on a cDNA or

genomic library by isolating cells which are decorated with a labeled form of the receptor. In a preferred embodiment, the technique uses the AR protein in an *in situ* assay for detecting AR ligands in tissue samples and whole organisms. In general, the RAP-*in situ* assay described below (for Receptor Affinity Probe) of Flanagan and Leder (see PCT publications WO 92/06220; and also Cheng et al. (1994) *Cell* 79:157-168) involves the use of an expression cloning system whereby an AR ligand is scored on the basis of binding to an AR/alkaline phosphatase fusion protein. In general, the method comprises (i) providing a hybrid molecule (the affinity probe) including the AR protein, or at least the ligand binding domain thereof, covalently bonded to an enzymatically active tag, preferably for which chromogenic substrates exist, (ii) contacting the tissue or organism with the affinity probe to form complexes between the probe and a cognate ligand in the sample, removing unbound probe, and (iii) detecting the affinity complex using a chromogenic substrate for the enzymatic activity associated with the affinity probe.

Furthermore, by making available purified and recombinant AR polypeptides, the present invention facilitates the development of assays which can be used to screen for drugs which are either agonists or antagonists of the normal cellular function of the subject angiogenin receptors. In a general sense, the assay evaluates the ability of a compound to modulate binding between an angiogenin receptor and a ligand, e.g., such as an angiogenin protein, that interacts with the receptor, or to mimic the effects of ligand engagement. Exemplary compounds which can be screened against such AR-mediated interactions include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries, such as isolated from animals, plants, fungus and/or microbes.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with a ligand. Accordingly, in an exemplary screening assay of the present invention, a reaction mixture is generated to include an AR polypeptide, compound(s) of interest, and a "target molecule", e.g., a protein, which interacts with the

AR polypeptide. Exemplary target molecules include ligands, such as angiogenin proteins, as well as other peptide and non-peptide interacting molecules. Detection and quantification of interaction of the AR polypeptide with the target molecule provides a means for determining a compound's efficacy at inhibiting (or potentiating) interaction between the AR and the target molecule. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, interaction of the AR polypeptide and target molecule is quantitated in the absence of the test compound.

Interaction between the AR polypeptide and the target molecule may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled AR polypeptides, by immunoassay, by chromatographic detection or use of a biosensor based on, e.g., surface plasmon resonance.

Typically, it will be desirable to immobilize either AR or the target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of AR to the target molecule, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/AR (GST/AR) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the cell lysates, e.g. an ^{35}S-labeled lysate, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of target molecule found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins and other molecules on matrices are also available for use in the subject assay. For instance, either AR or target molecule can be immobilized utilizing conjugation of biotin and streptavidin. For instance,

biotinylated AR molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with AR, but which do not interfere with the interaction between the AR and target molecule, can be derivatized to the wells of the plate, and AR trapped in the wells by antibody conjugation. As above, preparations of an target molecule and a test compound are incubated in the AR-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the target molecule, or which are reactive with AR protein and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the target molecule. To illustrate, the target molecule can be chemically cross-linked or genetically fused (if it is a polypeptide) with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diaminobenzidine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

For processes which rely on immunodetection for quantitating proteins trapped in the complex, antibodies against the protein, such as anti-AR antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the AR sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) J Biol Chem 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharamacia, NJ).

In another embodiment, interaction between the subject receptor and a potential ligand (e.g., a surrogate ligand) can be detected using surface plasmon resonance such as is currently carried out with "sensor chip" technology. See for example U.S. Patent 5,485,277; Shinohara et al. (1995) *J Biochem* (Tokyo) 117:1076-1082; Nice et al. (1993)

J Chromatogr 646:159-168; and Jonsson et al. (1991) *Biotechniques* 11: 620-627. Surface plasmon biosensors are basically sensitive refractometers that monitor changes in the optical state of a protein layer, in the case a layer of AR protein. This is accomplished, for example, by depositing a layer of receptor on top of a thin metal film evaporated onto the base of a TIR prism. When TM-polarized light in the prism is incident at the proper angle to excite surface plasmons, the TM-polarized light is attenuated drastically. In the presence of a target bioagent, such as the ligand, the thickness and surface plasmon resonance changes, thereby altering the angular position of the reflected light. By measuring the shift in the reflected light angle as a function of added test agent, the concentration of binding of a test agent to the receptor can be quantified.

An exemplary drug screening assav of the present invention includes the steps of (a) forming a reaction mixture including: (i) an angiogenin polypeptide, (ii) an AR polypeptide, and (iii) a test compound; and (b) detecting interaction of the angiogenin and AR polypeptides. A statistically significant change (potentiation or inhibition) in the interaction of the angiogenin and AR polypeptides in the presence of the test compound, relative to the interaction in the absence of the test compound, indicates a potential agonist (mimetic or potentiator) or antagonist (inhibitor) of angiogenin bioactivity for the test compound. The reaction mixture can be a cell-free protein preparation, e.g., a reconstituted protein mixture or a cell lysate, or it can be a recombinant cell including a heterologous nucleic acid recombinantly expressing the AR polypeptide.

Where the AR polypeptide participates as part of an oligomeric complex forming an angiogenin receptor, e.g., which complex includes other protein subunits, the cell-free system can be, e.g., a cell membrane preparation, a reconstituted protein mixture, or a liposome reconstituting the receptor subunits as an angiogenin receptor.

In yet another embodiment, the drug screening assay is derived to include a whole cell expressing an AR polypeptide. The ability of a test agent to alter the activity of the AR protein can be detected by analysis of the recombinant cell. For example, agonists and antagonists of the AR biological activity can by detected by scoring for alterations in growth or differentiation (phenotype) of the cell, expression of a reporter gene, and/or nuclear translocation of angiogenin (see for example Moroianu and Rirdon (1994) *PNAS* 91:1677-1681). General techniques for detecting each are well known, and will vary with respect to the source of the particular reagent cell utilized in any given assay. For the cell-based assays, the recombinant cell is preferably a metazoan cell, e.g., a mammalian cell, e.g., an insect cell, e.g., a xenopus cell, e.g., an oocyte. In other embodiments, the angiogenin receptor can be reconstituted in a yeast cell.

In an exemplary embodiment, a cell which expresses the AR protein, e.g, whether endogenous or heterologous, can be contacted with a ligand of the AR protein, e.g., an angiogenin protein, which is capable of inducing signal transduction from the receptor, and the resulting signaling detected either at various points in the pathway, or on the basis of a phenotypic change to the reagent cell. A test compound which modulates that pathway, e.g., potentiates or inhibits, can be detected by comparison with control experiments which either lack the receptor or lack the test compound. For example, visual inspection of the morphology of the reagent cell can be used to determine whether the biological activity of the targeted AR protein has been affected by the added agent.

In addition to morphological studies, change(s) in the level of an intracellular second messenger responsive to signaling by the AR polypeptide can be detected. For example, in various embodiments the assay may assess the ability of test agent to cause changes in phophorylation patterns, adenylate cyclase activity (cAMP production), GTP hydrolysis, calcium mobilization, and/or phospholipid hydrolysis (IP_3, DAG production) upon receptor stimulation. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, in cells contacted with an angiogenin polypeptide, candidate agonists and antagonists to AR-dependent angiogenin signaling can be identified.

The interaction of an angiogenin protein with an AR protein may set in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of AR-dependen. angiogenin signaling include urokinase type plasminogen activator (see, for example, Jimi et al. *supra*). By selecting transcriptional regulatory sequences from such target genes that are responsible for the transcriptional-regulation of these genes in response to angiogenin induction, and operatively linking such promoters to a reporter gene. the present invention provides a transcription based assay which is sensitive to the ability of a specific test compound to influence angiogenin signalling pathways.

In an exemplary embodiment, the step of detecting interaction of the angiogenin and AR polypeptides comprises detecting, in a cell-based assay, change(s) in the level of expression of a gene controlled by a transcriptional regulatory sequence responsive to signaling by the AR polypeptide. Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on

angiogenin signaling. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of AR-dependent angiogenin induction.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by AR-dependent induction with an angiogenin protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the angiogenin activity, with the level of expression of the reporter gene providing the angiogenin-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the inductive activity of the angiogenin protein.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug. Many reporter genes are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art. A reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein.

Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly

luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Accordingly, yet another embodiment of the subject drug screening assays of the present invention provides a recombinant cell, e.g., for carrying out certain of the drug screening methods above, comprising: (i) an expressible recombinant gene encoding a heterologous AR polypeptide whose signal transduction activity is modulated by binding to an angiogenin protein; and (ii) a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the signal transduction activity of the AR polypeptide. Still another aspect of the present invention provides a kit for screening test compounds to identify agents which modulate the binding of angiogenin proteins with an angiogenin receptor, including the above-referenced cell, a preparation of purified angiogenin polypeptide, and AR angiogenin.

Still another drug screening assay makes use of purified or semi-purified preparations of the subject angiogenin receptor by detecting changes in, for example, an associated enzymatic activity in response to ligand binding. For instance, in light of the observation that the subject receptor may be have an intrinsic tyrosine kinase activity, the activation (or inactivation) of that activity in response to ligand binding can be scored in a drug screening assay. For instance, Finn *et al.* (1989) *Biol Chem Hoppe Seyler* 370:559-564 describes the use of purified insulin receptor to detect, *inter alia*, the autophosphorylating activity of human insulin analogs. A similar approach can be taken with purified angiogenin receptor.

After identifying certain test compounds as potential modulators of one or more bioactivities of an AR protein (such as angiogenin binding), the practioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

Another aspect of the present invention relates to a method of inducing and/or maintaining a particular vascularization state, or inhibiting vascularization, by contacting the cells with an agent which modulates AR-dependent signal transduction pathways. Such agents can be compounds identified in the drug screening assays described above, as

well as various forms of the subject angiogenin receptor, antisense constructs, and other agents described herein as inhibitors or potentiators of the subject angiogenin receptor. For instance, an extracellular fragment of the receptor, e.g., one which retains angiogenin binding, can be used as an antiangiogenesis agent, e.g., in that it may compete with the native receptor for angiogenin binding.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped together as angiogenic dependent or angiogenic associated diseases. The present invention provides therapies directed to controlling the angiogenic processes in a manner which leads to the abrogation or mitigation of these diseases. For instance, in one embodiment the subject method can be used for treating undesired angiogenesis (e.g., hypervascularization or neovascularization) in a human or animal by administering a composition comprising an effective amount of an agent which inhibits angiogenin-mediated signalling by the subject angiogenin receptor.

Diseases associated with corneal neovascularization that can be treated according to the present invention include but are not limited to, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, trauma, rheumatoid arthritis, systemic lupus, polyarteritis, Wegeners sarcoidosis, scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graft rejection.

Diseases associated with retinal/choroidal neovascularization that can be treated according to the present invention include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticurn, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes,

toxoplasmosis, trauma and post-laser complications. Other diseases include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy, whether or not associated with diabetes.

Diseases associated with chronic inflammation can be treated by the compositions and methods of the present invention. Diseases with symptoms of chronic inflammation include inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, psoriasis, sarcoidosis and rheumatoid arthritis. Angiogenesis is a key element that these chronic inflammatory diseases have in common. The chronic inflammation depends on continuous formation of capillary sprouts to maintain an influx of inflammatory cells. The influx and presence of the inflammatory cells produce granulomas and thus, maintains the chronic inflammatory state. Inhibition of angiogenesis by the compositions and methods of the present invention would prevent the formation of the granulomas and alleviate the disease.

The compositions and methods of the present invention can be used to treat patients with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. Both Crohn's disease and ulcerative colitis are characterized by chronic inflammation and angiogenesis at various sites in the gastrointestinal tract. Crohn's disease is characterized by chronic granulomatous inflammation throughout the gastrointestinal tract consisting of new capillary sprouts surrounded by a cylinder of inflammatory cells. Prevention of angiogenesis by the compositions and methods of the present invention inhibits the formation of the sprouts and prevents the formation of granulomas.

Crohn's disease occurs as a chronic transmural inflammatory disease that most commonly affects the distal ileum and colon but may also occur in any part of the gastrointestinal tract from the mouth to the anus and perianal area. Patients with Crohn's disease generally have chronic diarrhea associated with abdominal pain, fever, anorexia, weight loss and abdominal swelling. Ulcerative colitis is also a chronic, nonspecific, inflammatory and ulcerative disease arising in the colonic mucosa and is characterized by the presence of bloody diarrhea.

The inflammatory bowel diseases also show extraintestinal manifestations such as skin lesions. Such lesions are characterized by inflammation and angiogenesis and can occur at many sites other than the gastrointestinal tract. The compositions and methods of the present invention are also capable of treating these lesions by preventing the angiogenesis, thus reducing the influx of inflammatory cells and the lesion formation.

Sarcoidosis is another chronic inflammatory disease that is characterized as a multisystem granulomatous disorder. The granulomas of this disease may form anywhere in the body and thus the symptoms depend on the site of the granulomas and whether the disease is active. The granulomas are created by the angiogenic capillary sprouts providing a constant supply of inflammatory cells.

The compositions and methods of the present invention can also treat the chronic inflammatory conditions associated with psoriasis. Psoriasis, a skin disease, is another chronic and recurrent disease that is characterized by papules and plaques of various sizes. Prevention of the formation of the new blood vessels necessary to maintain the characteristic lesions leads to relief from the symptoms.

Another disease which can be treated according to the present invention is rheumatoid arthritis. Rheumatoid arthritis is a chronic inflammatory disease characterized by nonspecific inflammation of the peripheral joints. It is believed that the blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state of rheumatoid arthritis.

Another disease that can be treated according to the present invention are hemangiomas, Osler-Weber-Rendu disease, or hereditary hemorrhagic telangiectasia, solid or blood borne tumors and acquired immune deficiency syndrome.

In particular, antiangiogenic agents identified by the present invention can be used to prevent angiogenesis in solid tumors. Angiogenesis is prominent in solid tumor formation and metastasis, and has been associated with several solid tumors, including those deriving from colon, breast , prostate and lung, as well as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is important include solid tumors, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas. Prevention of angiogenesis according to the present invention can halt the growth of these tumors and the resultant damage to the animal due to the presence of the tumor.

Angiogenesis is important in two stages of tumor metastasis. The first stage where angiogenesis stimulation is important is in the vascularization of the tumor which allows tumor cells to enter the blood stream and to circulate throughout the body. After the tumor

cells have left the primary site, and have settled into the secondary, metastasis site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis can lead to the prevention of metastasis of tumors and containing the neoplastic growth at the primary site.

It should be noted that angiogenesis has been associated with certain blood-born tumors such as leukemias, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and spleen. It is believed that angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia-like tumors. Treatment with antiangiogenic agents as described herein can accordingly be used as part of a treatment for such tumors.

Angiogenesis is also involved in normal physiological processes such as reproduction and wound healing. Angiogenesis is an important step in ovulation and also in implantation of the blastula after fertilization. Prevention of angiogenesis, e.g., with AR antagonists of the present invention, could be used to induce amenorrhea, to block ovulation or to prevent implantation by the blastula.

Angiogenic and antiangiogenic agents, e.g., which can be identified by use of the subject angiogenin receptor, can each be used under various circumstances in promoting effective wound healing. In wound healing, excessive repair or fibroplasia can be a detrimental side effect of surgical procedures and may be caused or exacerbated by angiogenesis. Adhesions are a frequent complication of surgery and lead to problems such as small bowel obstruction. In those instance, antiangiogenic agents may be opportunely applied to prevent such complications.

On the other hand, mitogenic effects that angiogenin receptor agonists is of particular interest given their potential stimulatory effect on collateral vascularization and angiogenesis. Angiogenic agents identified by use of the subject receptor proteins can be used to promote the development of a hemovascular network in a mammal or to accelerate wound healing. Such agents are also potential therapeutics for accelerating vascularization of prosthetic devices, as well as nerve regeneration and in healing of torn or traumatized fibrocartilage material.

It is presently believed that such agents useful in promoting hair growth, e.g., such as minoxidil, may work by increasing blood flow to the scalp. Accordingly, angiogenic agents identified according the assays herein may be of use in promoting hair growth, as for example in the treatment of alopecia.

Thus, in certain embodiments, therefor, the invention provides a method for treating undesired angiogenesis in a human or animal comprising the step of administering to the human or animal with the undesired angiogenesis a composition comprising an effective amount of an inhibitor of angiogenin-dependent AR function, e.g., such as a compound which competitively inhibits binding of angiogenin by the receptor (such as a small organic molecule, a peptide or the like, or a soluble extracellular fragment of the AR protein), an AR antisense, a AR kinase inhibitor or the like. The subject method can be useful in the treatment or prevention of such undesired angiogenic process as may be associated with retinal neovascularization, choroidal neovascularization, diabetic retinopathy, macular degeneration, corneal neovascularization, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections other than leprosy, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi's sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, trauma, rheumatoid arthritis, systemic lupus, polyarteritis, Wegeners sarcoidosis, scleritis, Steven Johnson's disease, radial keratotomy, sickle cell anemia, sarcoid, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis, chronic vitritis, Lyme disease, Eales disease, Bechets disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, post-laser complications abnormal proliferation of fibrovascular tissue, hemangiomas, Osler-Weber-Rendu, solid tumors, blood borne tumors, acquired immune deficiency syndrome, ocular neovascular disease, osteoarthritis, diseases caused by chronic inflammation, Crohn's disease, ulceritive colitis, tumors of rhabdomyosarcoma, tumors of retinoblastoma, tumors of Ewing sarcoma, tumors of neuroblastoma, tumors of osteosarcoma, leukemia, psoriasis, atherosclerosis, and pemphigoid.

Another aspect of the invention features transgenic non-human animals which express a heterologous AR gene of the present invention, and/or which have had one or more genomic AR genes disrupted in at least a tissue or cell-types of the animal. Accordingly, the invention features an animal model for developmental diseases, which animal has one or more AR allele which is mis-expressed. For example, an animal can be generated which has one or more AR alleles deleted or otherwise rendered inactive. Such

a model can then be used to study disorders arising from mis-expressed AR genes, as well as for evaluating potential therapies for similar disorders.

The transgenic animals of the present invention all include within a plurality of their cells a transgene of the present invention, which transgene alters the phenotype of the "host cell" with respect to regulation by the AR protein, e.g., of cell growth, death and/or differentiation. Since it is possible to produce transgenic organisms of the invention utilizing one or more of the transgene constructs described herein, a general description will be given of the production of transgenic organisms by referring generally to exogenous genetic material. This general description can be adapted by those skilled in the art in order to incorporate specific transgene sequences into organisms utilizing the methods and materials described herein and those generally known in the art.

Exemplification

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLE I

Materials used in the following experimental examples were obtained as follows. Human angiogenin (Met-1) was isolated from an *Escherichia coli* expression system (Shapiro et al., 1988, Anal. Biochem. 175: 450-461) and was provided by Dr. R. Shapiro (Harvard Medical School); the anti-human angiogenin monoclonal antibody 26-2F (Fett et al., 1995 Biochemistry 33: 5421-5427) was provided by Dr. K. A. Olson (Harvard Medical School); bFGF, aFGF, anti-aFGF polyclonal antibody, fibronectin, monomeric Avidin-Sepharose, Streptavidin-alkaline phosphatase, and Biodyne B nylon membranes were from Promega; methyl-^{3}H-thymidine (6.7 Ci/mmol) were from Du Pont/New England Nuclear; excellulose GF-5 desalting columns and Iodo-Beads iodination reagent were obtained from Pierce; (disulfosuccinimidyl)suberate (Sulfo-DSS) was obtained from CalBiochem; sulfosuccinimidyl-6-(biotinamido)hexanoate (Sulfo NHS-Biotin) was from Vector Laboratories; complete protease inhibitor cocktail tablets were from Boehringer

Mannheim; anti-bFGF monoclonal antibody, biotin, ribonuclease A (RNase A) and deoxyribonuclease I (DNase I) were obtained from Sigma.

EXAMPLE II

Cell cultures used in the following experimental examples are described as follows. Human umbilical venous (HUVE), umbilical arterial (HUAE), and microvascular (HME) endothelial cells were purchased from Cell Systems Corp. (CS) as primary cultures isolated from human umbilical veins, arteries, and human foreskin dermal tissues, respectively. HUAE and HME cells were cultured on CS-attachment factor-coated flasks in CS-Endothelial cell growth medium containing 10% fetal bovine serum (FBS). HUVE cells were cultured on fibronectin-coated dishes in human endothelial serum free medium (HE-SFM) (Gibco BRL/Life Technology) containing 20 ng/ml bFGF or on uncoated dishes but in HE-SFM + 10% FBS + 20 ng/ml bFGF. Cells between passages 3 and 15 inclusive were used for all experiments. Cell numbers were determined with a Coulter counter, and cell viability was measured by Trypan Blue exclusion assay.

EXAMPLE III

^{125}I-angiogenin was prepared as follows with the use of Iodo-beads. One Iodo-bead was added to 175 µl of 0.114 M phosphate buffer, pH 6.5, containing 0.5 mCi $Na^{125}I$ and incubated at room temperature for 5 min. Angiogenin, 50 µg in 25 µl H_2O was added and the mixture was incubated at room temperature for another 15 min. The reaction was terminated by removing the Iodo-bead, and iodinated angiogenin was separated from free iodine by a GF-5 desalting column equilibrated in 0.1 M phosphate buffer, pH 6.5. Fractions of 0.5 ml were collected and the radioactivity in each fraction was determined with a gamma counter.

EXAMPLE IV

^{3}H-thymidine uptake into endothelial cells was determined in the following manner. HUVE and HME cells were seeded in either 24-well plates or 35 mm dishes at a density of $6x10^3$ - $1.5x10^4$ cells/cm^2 and cultured in HE-SFM containing 10% FBS and 20 ng/ml bFGF at 37 ^{0}C under humidified air containing 5% CO_2. Six replicates were used for each sample. After 24 hr, the cells were washed 3 times with prewarmed HE-SFM and serum-starved in HE-SFM for 18 hr. The culture medium was removed and the

cells were incubated in HE-SFM with test samples in the presence of 1 μCi/ml ^{3}H-thymidine for 14 hr. At the end of the incubation, the cells were washed 3 times with phosphate buffered saline (PBS), precipitated with 10% trichloroacetic acid at RT for 30 min, washed 2 times with ethanol and solubilized with 0.2 M NaOH + 0.2% SDS. After neutralization with 1/5 volume of 1 N HCl, the radioactivity was determined by liquid scintillation counting.

EXAMPLE V

Cell proliferation was determined in the following manner. HUVE and HME cells were seeded in either fibronectin- or CS-attachment factor-coated 35 mm dishes in HE-SFM at 4-8x10^3 cells/cm^2. Test samples (10 μl) were added immediately after the cells were seeded. When combinations of samples were tested, they were premixed and always adjusted to a final volume of 10 μl with HE-SFM before addition to the cells. The cells were incubated at 37 ^{0}C in humidified air containing 5% CO_2 for 48 hr. At the end of this time, the medium was aspirated, and the cells were washed once with 1 ml PBS and detached with 0.25 ml of trypsin-versene (0.05%) solution. Cell numbers were determined with a Coulter counter.

EXAMPLE VI

As shown in Table 1 below, angiogenin stimulates ^{3}H-thymidine incorporation and cell proliferation of human endothelial cells under defined conditions described in Examples IV and V. In 5 and 15 independent experiments, 1 μg/ml angiogenin stimulated HUVE cells to increase ^{3}H-thymidine incorporation and proliferation by 33 and 34%, respectively. In 14 and 9 independent experiments with HME cells, angiogenin stimulated an average increase of 19 and 25% in ^{3}H-thymidine incorporation and cell proliferation, respectively. Student-Fisher's *t*-test showed that the increases of ^{3}H-thymidine incorporation and cell proliferation induced by angiogenin in both HUVE and HME cells are statistically significant, as indicated by P values.

Table 1

	^{3}H-thymidine incorporation			Cell proliferation		
Cells	% increase	P value	n	% increase	P value	n
HUVE	33±4.7	<0.005	5	34±3.0	<0.0005	15
HME	19±2.9	<0.005	14	25±2.3	<0.0005	

EXAMPLE VII

Angiogenin-stimulated cell growth was concentration dependent as shown by Fig. 1 which is a graph showing experimental results of dose-dependent stimulation of ^{3}H-thymidine incorporation and proliferation of endothelial cells by angiogenin. For ^{3}H-thymidine incorporation (left panel), $1x10^4$ of HUVE (solid lines) and HME (dashed lines) cells were used per well in 24-well plates ($5x10^3$ cells/cm^2) with 6 replicates for each sample. For cell proliferation assays, $5x10^4$ cells were used per 35 mm dish ($5x10^3$ cells/cm^2) with 3 replicates for each sample. Data shown are the percent increases over controls.

At 10 and 100 ng/ml, angiogenin stimulated a 14% and 48% and a 15% and 30% increase of ^{3}H-thymidine incorporation in HUVE and HME cells, respectively (Fig. 1, left panel). Raising the angiogenin concentration to 1 μg/ml enhanced ^{3}H-thymidine uptake by 54% and 32% for HUVE and HME cells, respectively, not significantly different from results seen at 100 ng/ml. A similar pattern of concentration dependency was observed for angiogenin-stimulated cell proliferation (Fig. 1, right panel). Almost no proliferation occurred when cells were cultured in HE-SFM in the absence of angiogenin. In the presence of 10, 100 and 1000 ng/ml of angiogenin, proliferation was increased by 29%, 42%, and 52% for HUVE cells, and by 8%, 15%, and 31% for HME cells, respectively. A 30% increase of HME cell proliferation was observed with 10 μg/ml angiogenin in a separate experiment, indicating that angiogenin activity in HME cells reached saturation at 1 μg/ml.

EXAMPLE VIII

As shown in Table 2 below, angiogenin-induced ^{3}H-thymidine incorporation and cell proliferation of HUVE cells were inhibited by 26-2F, an anti-angiogenin monoclonal antibody (Fett et al., 1995, Biochemistry 33: 5421-5427), but not by CCL130, a non-immune control antibody. HUVE cells were plated at 15000 cells per 35 mm culture dish (1500 cells/cm^2). The effect of 20 ng/ml angiogenin, which induced a 33% increase in ^{3}H-thymidine incorporation and a 16% increase in cell proliferation, was completely inhibited by 10 μg/ml of 26-2F. Mixtures of angiogenin with 26-2F had no stimulating effect but with CCL 130 (10 μg/ml) there was a 37% and a 14% increase, respectively.

Table 2

Assays	Control	Angiogenin	Angiogenin + 26-2F	Angiogenin + CCL 130
^{3}H-thymidine				
(cpm)	11700±1300	15600±1100	11500±1200	16000±700
(%)	100	133	98	137
Proliferation				
(cell number)	14800±800	17200±300	15100±1000	16800±200
(%)	100	116	102	114

EXAMPLE IX

Cell density was the most important factor that influenced angiogenin-induced ^{3}H-thymidine incorporation and cell proliferation. Endothelial cells responded to angiogenin only when they were in sparse culture. The responsiveness diminished when the cell density exceeded $2x10^4$ cells/cm^2. On the other hand, cells cannot be too sparse (<10^3/cm^2) due to the resulting high signal/noise ratio. The optimal density for ^{3}H-thymidine incorporation and cell proliferation assays was $6x10^3$ - $1.5x10^4$ and 4 - $8x10^3$ cells/cm^2, respectively.

The type of attachment factor employed was another critical item. In the ^{3}H-thymidine incorporation assay best results were obtained without the use of any attachment factor. However, for successful cell proliferation assays culture dishes should be coated with fibronectin or the attachment factors from CS.

As shown in Table 3 below, angiogenin activity was not masked by exogenous bFGF. HUVE and HME cells, at $7x10^4$ and $5x10^4$ per 35 mm dish, were stimulated by angiogenin (1 µg/ml), bFGF (2 ng/ml) or a mixture of the two, respectively. Angiogenin alone stimulated 33 and 16% increases, and bFGF alone stimulated 182 and 36% increases in HUVE and HME cell proliferation, respectively. A mixture of angiogenin and bFGF stimulated 365 and 62% increases, which were 29 and 19% greater than for samples treated just with bFGF. The percent increase stimulated by angiogenin in the presence of bFGF was very close to that seen in its absence. There was no synergistic effect when the cells were stimulated simultaneously with bFGF and angiogenin. They also did not antagonize each other in the ^{3}H-thymidine incorporation assay. The results

lead to the conclusion that angiogenin and bFGF stimulate cell growth independently, probably through interaction with distinct cellular receptor proteins.

Table 3

Cells	Control	Angiogenin	bFGF	bFGF + Angiogenin
HUVE				
(cell number)	69900±1000	93000±4000	197000±3600	255000±5000
(%)	100	133	282	365
HME				
(cell number)	49900±1400	57900±1400	67700±1600	80800±1100
(%)	100	116	136	162

As shown in Table 4 below, angiogenin-stimulated proliferation of HUVE cells was not inhibited by RNase A, a close homolog of angiogenin but one which is not angiogenic. Experiments were carried out in T-25 flasks containing $1.5x10^5$ cells per flask ($6x10^3$ cells/cm^2). Three flasks were used for each sample. Concentrations of angiogenin and RNase A were 1 and 10 µg/ml, respectively. The percent increases induced by 1 µg/ml angiogenin in the absence or presence of 10 µg/ml RNase A were 25 and 21%, respectively, while 10 µg/ml RNase A itself had no effect on cell proliferation under these conditions. The fact that RNase A is not active under the same conditions and did not inhibit angiogenin-induced ^{3}H-thymidine incorporation and cell proliferation indicated that the action of angiogenin in endothelial cells is specific.

Table 4

	Control	Angiogenin	RNase A	Angiogenin + RNase A
Cell number	192300±600	240200±1900	197700±300	232300±1000
%	100	125	103	121

Endothelial cells are known to secrete aFGF and bFGF (Folkman et al., 1988, Am. J. Pathol. 130: 393-400). We therefore investigated the effects of endogenous FGFs on the activity of angiogenin. HUVE and HME cells were plated at $1.6x10^4$ cells per well in 24-well plates ($8x10^3$ cells/cm^2). Concentrations of angiogenin, anti-aFGF and anti-bFGF

antibody were 1, 10 and 10 μg/ml, respectively. As shown in Table 5 below, an anti-aFGF antibody had little effect, while an anti-bFGF monoclonal antibody significantly reduced spontaneous ^{3}H-thymidine incorporation of both HME and HUVE cells. These results suggest a critical role of endogenous bFGF but not aFGF in survival and minimum growth of endothelial cells under serum free conditions. Nevertheless, the angiogenin-induced ^{3}H-thymidine incorporation in both cell types was not inhibited by anti-aFGF antibodies, anti-bFGF antibodies or a combination of the two. The increases stimulated by angiogenin relative to the corresponding controls in the presence of one, the other or both antibodies were 14, 21, and 30% in HUVE cells and 15, 27, and 37% in HME cells, respectively. These results were not significantly different from those obtained in the absence of anti-FGF antibodies, 34 and 24% for HUVE and HME cells, respectively.

Table 5

	Control		aFGF IgG		bFGF IgG		aFGF IgG + bFGF IgG	
Angiogenin	-	+	-	+	-	+	-	+
Cells								
HUVE								
(cpm)	4400 ±200	5900 ±50	5100 ±100	5800 ±100	3400 ±600	4100 ±500	3000 ±200	3900 ±100
(%)	100	134	100	114	100	121	100	130
HME								
(cpm)	9700 ±300	12000 ±300	10500 ±400	12100 ±500	5900 ±300	7500 ±400	5900 ±800	8100 ±400
(%)	100	124	100	115	100	127	100	137

EXAMPLE X

The response of endothelial cells to angiogenin in ^{3}H-thymidine incorporation and cell proliferation indicates that a cellular receptor protein for angiogenin must be expressed under such conditions. To identify this receptor, ^{125}I-labeled angiogenin was used to bind to the surface of endothelial cells under sparse culture conditions where they exhibit a positive response in both assays. Sulfo-DSS, a water soluble, membrane impermeable, bifunctional chemical crosslinker, was used to crosslink ^{125}I-angiogenin to its receptor. It is known that with subconfluent endothelial cells angiogenin binds to cell

surface actin and that the angiogenin-actin complex dissociates from the cell surface (Hu et al., 1993, Proc. Natl. Acad. Sci. USA 90 1217-1221). If actin was to be expressed on the endothelial cell surface under the conditions used above, it might prevent angiogenin from binding to other cell surface receptors especially when low concentrations of angiogenin or short incubation times are employed. Therefore, the cells were first incubated with 50 ng/ml of ^{125}I-angiogenin to deplete them of any cell surface actin. After the first incubation, the cells were reincubated with 50 ng/ml of fresh ^{125}I-angiogenin and then crosslinked by Sulfo-DSS.

Specifically, endothelial cells (HUVE, HUAE, HME), seeded at $5x10^3$ cells/cm^2, were cultured in HE-SFM + 0% FBS + 20 ng/ml bFGF at 37 ^{0}C under 5% humidified CO_2 for 24 hr, and starved in HE-SFM for another 24 hr. The cells were then cooled to 4 ^{0}C, washed twice with PBS, and incubated with 50 ng/ml ^{125}I- angiogenin in PBS at 4 ^{0}C for 30 min. At the end of this time, the supernatant was removed and 1 ml of 50 ng/ml fresh ^{125}I-angiogenin in PBS was readded to the cells and incubated at 4 ^{0}C for another 30 min. Unbound ^{125}I-angiogenin from the second incubation was removed by washing 3 times with PBS. Bound ^{125}I-angiogenin was crosslinked to the cell surface by treatment with 0.1 mM Sulfo-DSS in PBS at 4 ^{0}C for 10 min. Unreacted Sulfo-DSS was quenched by 5 mM Tris-HCl, pH 7.5. The cells were then washed twice with PBS and solubilized in 100 µl of 1xSDS-PAGE sample buffer. The entire sample was subject to SDS-PAGE and autoradiography. In competition experiments, the competitors were premixed with ^{125}I-angiogenin and were present in both incubations.

As shown in Fig. 2, left panel, SDS-PAGE and autoradiography revealed a crosslinked band of approximately 180 kDa with HUVE (lane A), HUAE (lane B) and HME (lane C) cells, indicating that a ~ 170 kDa angiogenin binding protein is expressed on the surface of 3 types of endothelial cells. In this experiment ^{125}I-angiogenin-actin complex was not observed since only cell monolayers were analyzed. Further, Sulfo-DSS is known to be an inefficient crosslinker for actin-angiogenin (Hu et al., 1993, supra). Binding of ^{125}I-angiogenin to the 170 kDa protein (Fig. 2, right panel, lane A) was inhibited by a 10-fold excess of unlabeled angiogenin (lane C), but not by RNase A (lane B), indicating that the 170 kDa protein is specific for angiogenin.

Expression of the 170 kDa angiogenin receptor is correlated with the activity of angiogenin in ^{3}H-thymidine incorporation and cell proliferation assays. No crosslinked band was detected when cell density exceeded $2x10^4$ cells/cm^2. These data suggest that the 170 kDa protein may serve as a functional receptor for angiogenin and mediate angiogenin-stimulated proliferation of endothelial cells.

It appears that the 170 kDa angiogenin receptor and actin are not expressed concurrently on the endothelial cell surface. The 170 kDa angiogenin receptor and actin may participate in certain specific cellular functions at different stages in the process of angiogenin-induced angiogenesis. Binding of angiogenin to cell surface actin has been demonstrated to result in activation of a cell-associated protease system and cell invasion (Hu et al., 1994, supra). After the cells are activated and start to migrate and invade the basement membrane, the local density of the cells in the vicinity of the migrating cells decreases and this may trigger the expression of the 170 kDa angiogenin receptor on the remaining adjacent cells. These cells become responsive to stimulation by angiogenin and will therefore divide to fill the space created by the migrating cells. Such density-dependent receptor expression may regulate the angiogenin-induced growth of the new capillary network.

EXAMPLE XI

The binding constant between angiogenin and its cell surface receptor was determined in the following manner. HUVE cells were cultured under the angiogenin-responsive conditions as described in Example X. The cells were cooled to 4 °C, washed three times with PBS and incubated with various concentration of ^{125}I-angiogenin in PBS at 4 °C for 30 min. At the end of incubation, the medium was removed and the cells were washed 3 times with PBS. The medium and the washing solution were combined and the radioactivity was counted as free ^{125}I-angiogenin. The cell monolayers were washed once with PBS + 0.5 M NaCl followed by washing with PBS 2 times. Radioactivity in these washing solutions was counted as nonspecifically bound ^{125}I-angiogenin. The cells were then solubilized with 62.5 mM Tris-HCl, pH 6.8, containing 3% SDS and 5% 2-mercaptoethanol. The radioactivity in the cell solubilizate was counted as the bound ^{125}I-angiogenin. The left panel of figure 3 the curve of concentration dependency of ^{125}I-angiogenin binding to HUVE cells. Scatchard analysis (Fig.3, right panel) of the binding data showed that ^{125}I-angiogenin binds to HUVE cells with an apparent K_d of 5.9 nM.

EXAMPLE XII

Angiogenin undergoes nuclear translocation in endothelial cells and this process of nuclear translocation is necessary for its angiogenic activity (Moroianu and Riordan, 1994, Proc. Natl. Acad. Sci. USA, supra; Moroianu and Riordan, 1994, Biochem.

Biophys. Res. Commun., supra). The correlation of nuclear translocation of angiogenin and the expression of the 170 kDa angiogenin receptor was established in the following manner. HUVE cells, at densities between $5x10^3$ and $2x10^4$ cells/cm^2, were cultured in HE-SFM in the presence of 20 ng/ml bFGF at 37 °C for 24 hr under 5% of humidified CO_2. The medium was aspirated and the cells were washed twice with prewarmed (37 °C) HE-SFM. ^{125}I-angiogenin was added to the cells at a final concentration of 0.2 µg/ml in HE-SFM and incubated at 37 °C for 30 min. At the end of the incubation, the medium was removed and the cells were washed three times with PBS followed by washing 3 times with 10 mM phosphate, pH 3.0, containing 150 mM NaCl. The cells were then lysed with 10 mM HEPES, pH 7.5, containing 0.5% Triton X-100 and 1 x complete protease inhibitor cocktail mixture. The lysates were collected and the nuclei monolayers were washed with PBS three times and collected by solubilizing in 62.5 mM Tris-HCl, pH 6.8, containing 3% SDS and 5% 2-mercaptoethanol. The radioactivity in each subcellular fraction was counted with a Gamma counter. Table 6 showed that the amount of angiogenin accumulated in the nucleus decreased when the cell density increased. These results correlate with the fact that expression of the 170 kDa angiogenin receptor decreases as cell density increases, implying that the 170 kDa receptor is involved in the process of nuclear translocation of angiogenin.

Table 6

Density (cells/cm^2)	Total cell number	Angiogenin in nucleus (cpm)	cpm per 10^3 cells
5×10^3	5×10^4	3228±186	640
1×10^4	1×10^5	2532±302	250
1.5×10^4	1.5×10^5	1944±202	130
2×10^4	2×10^5	1318±7	65

EXAMPLE XIII

Table 7 shows the effect of pretreatment of anti-angiogenin monoclonal antibody on ^{3}H-thymidine incorporation in HUVE cells stimulated by angiogenin. The effect of the endogenous angiogenin secreted by the cells was neutralized by incubating the cells with 60 µg/ml of 26-2F for 6 hr. Cells were washed 3 times with HE-SFM and stimulated with 1 µg/ml angiogenin in HE-SFM containing 1 µCi/ml ^{3}H-thymidine for 18 hr. Incorporation of ^{3}H-thymidine in TCA-insoluble materials were determined as described in Example IV. Results showed that pretreatment of the cells with anti-angiogenin antibody enhances the response of HUVE cells to exogenous angiogenin in ^{3}H-thymidine incorporation assay. Without pretreatment, 1 µg/ml of angiogenin stimulated a 39%

increase of ^{3}H-thymidine incorporation. However, after the pretreatment of 26-2F, the same concentration of angiogenin stimulated a 74% increase of ^{3}H-thymidine incorporation. These results indicated that either the expression of the angiogenin receptor or the reactivity of the receptor with angiogenin is regulated by endogenous angiogenin as well as by cell density and by the use of attachment factors.

Table 7

	Control	Angiogenin
Without pretreatment of 26-2F	3800±400	5300±500
(%)	100	139
With pretreatment of 26-2F	3500±300	6100±400
(%)	100	174

Table 8 showed that fetal bovine serum (FBS) completely inhibited the activity of angiogenin. HME cells were plated at 1 x 10^4 cells per well in 24-well plates (5 x 10^3 cells/cm^2). FBS was at 1% and was present during both starvation and stimulation. FBS itself stimulated a 67% increase in ^{3}H-thymidine incorporation into HME cells. When angiogenin was added in the presence of 1% FBS, the stimulation, 67%, was the same as in the absence of angiogenin. In contrast, the same concentration of angiogenin added to the cells in HE-SFM resulted in a stimulation of 19%.

Table 8

	Control	Angiogenin	FBS	FBS+angiogenin
cpm	5700±60	6800±200	9500±400	9500±700
(%)	100	119	167	167

EXAMPLE XIV

Isolation and purification of the angiogenin receptor from the endothelial cell surface was accomplished in the following manner. HUVE cells were cultured under angiogenin-responsive conditions as described and their surface molecules were biotinylated and enriched by affinity chromatography on monomeric Avidin-Sepharose. After successive adsorption by DNase I-Sepharose and RNase A-Sepharose to remove actin and other nonspecific binding proteins, the biotinylated angiogenin receptor was purified by affinity chromatography on an angiogenin-Sepharose column. The final

product was analyzed by SDS-PAGE and detected with Streptavidin-alkaline phosphatase after transfer to a Biodyne B nylon membrane.

Specifically, HUVE cells, at passage 5, were plated at $5x10^3$ cells/cm^2 in a T162 flask and cultured in HE-SFM + 10% FBS + 20 ng/ml bFGF at 37 ^{0}C for 24 hr. The culture medium was removed and the cells were washed once with prewarmed HE-SFM and incubated in HE-SFM for another 18 hr. The cells were cooled at 4 ^{0}C for 10 min, washed 3 times with cold PBS and biotinylated by incubating with 15 ml of 0.5 mg/ml Sulfo-NHS-Biotin in PBS at 4 ^{0}C for 30 min with gentle shaking. Unreacted reagent was removed by washing the cells 3 times with PBS. Biotinylated membrane molecules were solubilized with 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.2, containing 1x complete protease inhibitor cocktail at 4 ^{0}C for 30 min. The solubilizates were centrifuged to remove cell debris and subjected to chromatography on a monomeric Avidin-Sepharose column. Biotinylated molecules were eluted from the column by 0.1 M biotin in 0.1 M phosphate buffer, pH 7.2, and were successively adsorbed by DNase I-Sepharose and RNase A-Sepharose. The flowthrough fraction was then applied to an angiogenin-Sepharose column and washed with 5 volumes of H_2O. The bound materials were eluted from the column with 0.1% trifluoroacetic acid, lyophilized, and analyzed by SDS-PAGE and western blotting with alkaline phosphatase labeled Streptavidin.

As shown in Fig. 4, a single band of approximately 170 kDa was observed, consistent with the molecular weight estimation from the crosslinking experiments. Approximately 10 ng protein was obtained from cells grown in a single T162 flask.

EXAMPLE XV

A partial amino acid sequence of a 55 kDa fragment of the angiogenin receptor was obtained in the following manner. In a scaled-up preparation of the 170 kDa angiogenin receptor, a 55 kDa fragment was also isolated from HUVE cells by methods described in Example XIV. The 55 kDa fragment was separated from the 170 kD protein by SDS/PAGE and the products were visualized by Coomasssie Blue staining. After destaining with 10% ethanol and 7.5% acetic acid, a gel slice containing the 55 kDa fragment was cut and transferred to an Eppendorf tube. The gel slice was washed with water three times over a period of 3 hr, and the peptide was extracted with 150 µl of 0.1 M sodium acetate, pH 8.5, containing 0.1% SDS at 37 C for 14 hr. After centrifugation to remove the gel debris, the extract was applied directly to a Millipore Prosequencer membrane for amino acid sequence analysis (Laursen et al., 1989, Methods in Protein

Sequence Analysis (Wittman-Leibold B., ed.), Springer-Verlag, Berlin, pp 61-68). Water soluble carbodiimide was used for covalent attachment of the sample to an aryl amine PVDF membrane disc. The amino acid assignment for the first 15 residues was Tyr-Ala-Ile-Arg-Ala-Ser-Asn-Ile-Glu-Lys-Pro-Leu-Gly-Tyr-Phe (SEQ ID No. 1). A search for identities with the Protein Identification Resource at the National Center for Biotechnology Information revealed 73% identity with residues 1340-1354 of ROS 1 transmembrane tyrosine kinase (Birchmeier et al., 1990, Proc. Natl. Acad. Sci. USA 87: 4799-4803).

An internal amino acid sequence of the 170 kDa receptor was obtained in the following manner. The 170 kDa protein was isolated by methods described in Example XIV and peptides were produced by Endoproteinase Glu-C digestion in 50 mM ammonium acetate, pH 4.0, at room temperature for 5 min. The peptides were separated by reversed-phase HPLC on a Delta-Pak C_{18} column (Waters Corp.). A single seemingly well-resolved peak was selected for amino acid sequence analysis (Laursen et al., 1989, supra). The amino acid assignment for the first 20 residues was Lys-Gly-Val-Thr-Val-Leu-Ile-Val-Asn-Gln-Gln-Glu-Phe-Xaa-Arg-Ala-Leu-Ala-Ala-Phe (SEQ ID No. 2). A search for identity revealed 55% identity with residues 1894-1915 with a gap of 2 residues at positions 1908 and 1909 of ROS 1 transmembrane tyrosine kinase (Birchmeier et al., 1990, supra).

An another internal amino acid sequence of the 170 kDa receptor was obtained in the following manner. The tryptic peptides of the 170 kDa receptor were produced by trypsin digestion in 50 mM Tris-HCl, pH 7.6, at 37 °C for 2 hr and separated by reversed-phase HPLC on a Delta-pak C18 column. A single seemingly well-resolved peak was analyzed for amino acid sequence (Laursen et al., 1989, supra). The amino acid assignment for the first 9 residues was His-Trp-Tyr-Pro-Asn-Tyr-Leu-Gly-Met (SEQ ID No. 3). BLAST search for identities revealed that this sequence is not homologous to any known sequences that have been deposited to the data bank located in the National Center for Biotechnology Information.

EXAMPLE XVI

A cDNA library of HUVE cells was constructed in the following manner. HUVE cells were cultured at a cell density of 5 x $10^3/cm^2$, in HE-SFM containing 10% FBS and 20 ng/ml of bFGF at 37 °C under 5% CO_2 for 24 hr. The cells were washed 3 times with prewarmed HE-SFM and serum-starved in HE-SFM for another 24 hr. Cell culture

medium was removed and the cells were washed once with PBS and collected by scraping with a rubber cell scraper. Experiments described in examples IX and X have demonstrated that the 170 kDa angiogenin receptor was expressed maximally under these conditions. mRNA from 4.7 x 10^7 HUVE cells was extracted with a Poly A Tract System 1000 Kit from Promega Corp. (Chirgwin et al., 1979, Biochemistry 18: 5294-5299). A total of 23 μg mRNA was obtained and 2 μg of which was used for cDNA synthesis with the Universal Riboclone cDNA synthesis system from Promega Corp., which is based on the methods of Okayama and Berg (Okayama and Berg, 1982, Mol. Cell Biol. 2: 161-170) with modifications by Gubler and Hoffman (Gubler and Hoffman, 1983, Gene 25, 263-269). First strand synthesis was achieved by Avian Myeloblastosis Virus reverse transcriptase and an oligo (dT) primer, followed directly by second strand replacement synthesis with RNase H and DNA polymerase I. The double-stranded cDNA were flushed at both ends by T_4 DNA polymerase, fractionated to remove small (<500 bp) fragments, and ligated with an *EcoR* I adaptor at both ends. A total of 300 ng of cDNA was obtained. A cDNA library was constructed by inserting the synthesized cDNA into the *EcoR* I site of λgt 11 vector (Watson and Jackson, 1985, DNA Cloning, Vol 1 (ed. Glove DM): IL, Oxford, 79-88). The *EcoR* I site on λgt 11 is located within the *Lac* Z gene, upstream from the β-galactosidase translation termination codon. Because insertion disrupted the *Lac* Z gene, recombinant phages were identified as colorless plaques in *E. Coli.* Strain Y1090 in the presence of X-gal and IPTG. The cDNA library prepared as described above had a titer of 3.8 x 10^7 pfu/ml.

EXAMPLE XVII

Oligonucleotide probes to be used for screening the cDNA encoding the 170 kDa angiogenin receptor was obtained in the following manner. Since SEQ ID No. 1 and No. 2 showed 73 and 55% identity, respectively, with residues 1340-1354 and 1894-1915 of ROS 1 transmembrane tyrosine kinase (Birchemeier et al., 1990 supra), and the SEQ ID No. 3 is not homologous to any known sequences. The following strategy was used to obtain the cDNA for angiogenin receptor. The cDNA library of HUVE cells was first screened with a ROS 1 cDNA probe. Clones that are positive to ROS 1 were then screened with an oligonucleotide probe that was designed based on the SEQ ID No. 3. Clones that are positive both to ROS 1 cDNA and the oligonucleotide probe would be most likely the candidates for angiogenin receptor. The ROS 1 cDNA probe was prepared from a phagemid clone of human avian UR2 sarcoma virus oncogene (v-ros) homolog ROS 1 (ATCC 65238). The plasmid DNA was isolated by using a Wizard Midiprep DNA

Isolation System from Promega Corp. (Brinboin, 1983, Meth. Enzymol, 100, 243) and the cDNA insert of 3.62 kb was excised by *Not* I digestion and purified by agarose gel electrophoresis. The isolated cDNA insert was labeled radioactively by a Nick Translation Kit from Boehringer Mannheim (Rigby et al., 1977, J. Mol. Biol. 113: 237-251) to give a specific activity of ~ 1 x 10^9 cpm/μg. The degenerated oligonucleotide probe based on SEQ ID No. 3 was chemically synthesized. Since one amino acid can be encoded by more than one codons, e.g. CCA, CCC, CCG, and CCU all encode proline, the synthesized probe has the following sequences: CA(U,C)UGGUA(C,U)CC(A,C,G,U)AA(C,U)UA, which is a mixture of 32 nucleotide. This probe was radioactively labeled at the 5'-end by [γ^{32}P]ATP and T_4 polynucleotide kinase. The 5'-end labeled Oligo probe No. 1 has a specific activity of 4-8 x 10^8 cpm/μg.

EXAMPLE XVIII

Screening the library for the cDNA encoding the 170 kDa angiogenin receptor was performed in the following manner. Three plates (150 mm), each containing 1 x 10^5 recombinant phages in the top agarose layer, were prepared and incubated at 37 °C for 4 hr until the plaques were pinpoint in size. The plates were then put at 4 °C for 1 hr to harden the agarose. DNAs from the plaques were transformed to NYTRAN butterfly membranes (Schleicher & Schuell) at room temperature for 10 min. Two replicates were prepared from each plate. The membranes were dried at room temperature for 10 min and washed once for 2 min successively with 0.2 M NaOH containing 1.5 M NaCl, 0.4 M Tris-HCl, pH 7.6, containing 0.3 M NaCl and 30 mM sodium citrate, and 30 mM sodium citrate, pH 7.0, containing 0.3 M NaCl. The membranes were baked at 80 °C under vacuum for 2 hr and prehybridized at 68 °C for 3 hr in a buffer system containing 75 mM sodium citrate, pH 7.0, 0.75 M NaCl, 10% dextran sulfate, 0.3% tetrasodium pyrophosphate, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 100 ng/ml sonicated salmon sperm DNA. ^{32}P-labeled ROS 1 cDNA probe was denatured by boiling and snap-cooling on ice and added to the prewarmed hybridization solution to a final concentration of ~ 1 x 10^6 cpm/ml. The membranes were incubated in this solution at 68 °C overnight with gentle shaking. After hybridization, the membranes were washed at room temperature twice for 5 min in 30 mM sodium citrate, pH 7.0, containing 0.3 M NaCl, followed by washing in 3 mM Sodium citrate, pH 7.0, containing 30 mM NaCl and 0.1% SDS once for 5 min at room temperature, twice for 15 min at 42 °C and twice at 68 °C for 15 min. Autoradiography identified 10 clones that appeared positive in both

replicates. They were selected and screened further with the same probe (ROS 1 cDNA) until all the plaques in the plate gave positive signals.

The lamda phage DNAs from the 10 clones were prepared with the use of Wizard lamda DNA Isolation Kit from Promega Corp. The insert cDNAs were excised by *EcoR* I digestion. The size of the inserts from clones 1 to 10 were determined by agarose electrophoresis to be 1.4, 3.5, 0.8, 3.0, 1.5, 0.7, 2.1, 1.7, 2.0, and 3.0 kb, respectively.

Southern blotting of the insert cDNAs with ^{32}P-labeled ROS 1 was performed to confirm that the clones selected by *in situ* hybridization are not the results of non-specific interaction. Products of *EcoR* I digestion of lamda DNAs isolated from the 10 clones were separated by agarose gel electrophoresis and denatured by shaking gently in 0.25 M HCl for 20 min at room temperature. The gel was washed with water for 2 min and incubated in 0.5 M NaOH and 1.5 M NaCl at room temperature for 30 min. DNAs were transferred to a Biodyne B nylon membrane by capillary absorption in 0.3 M sodium citrate + 3 M NaCl overnight. The membrane was baked at 80 °C for 30 min to fix the DNAs and incubated with ^{32}P-labeled ROS 1 cDNA (final concentration 5 x 10^5 cpm/ml) in 15 mM phosphate buffer, pH 7.7, containing 10% PEG, 7% SDS, 0.3 M NaCl, and 1.5 mM EDTA at 65 °C overnight. The membrane was washed once at room temperature for 15 min with 30 mM sodium citrate, pH 7.0, containing 0.3 M NaCl and 0.1% SDS, once at 65 °C for 20 min with 1.5 mM sodium citrate, pH 7.0, containing 30 mM NaCl and 0.1% SDS. Autoradiography revealed that all the insert cDNA hybridized to ROS 1 cDNA, whereas the vector DNA did not.

^{32}P-labeled Oligo probe No. 1 was used in another Southern blotting experiment to identify the double positive clones. DNAs were transferred to the nylon membrane and fixed as described above. After incubation at 48 °C for 2 hr in a prehybridization solution that is 10 mM phosphate, pH 6.8, containing 3M TMAC (tetramethylammonium chloride), 1 mM EDTA, 0.5% SDS, 0.1% Ficoll, 0.1% Polyvinyl pyrrolidone, 0.1% BSA, and 100 ng/ml denatured salmon testes DNA, the membrane is hybridized with ^{32}P-labeled Oligo probe No. 1 (final concentration: 10^4/ml) in the same solution at 48 °C for 40 hr. The membrane was washed three times at 50 °C for 1 hr with 50 mM Tris-HCl, pH 8.0, containing 3 M TMAC and 0.2% SDS, and twice at room temperature with 30 mM sodium citrate, pH 7.0, containing 0.3 M NaCl, and 0.1% SDS. Autoradiography showed that the cDNA insert from clone No. 8 hybridized with Oligo probe No. 1.

EXAMPLE XIX

The nucleotide sequence of the cDNA insert from clone 8 was determined in the following manner. The lamda DNA from clone 8 was isolated as the template for PCR reaction. Two oligonucleotides that match the sequences flanking the *EcoR* I insertion site of λgt 11 genome were synthesized as the forward and reverse primers, respectively. The primers were end-labeled by ^{32}P with the use of T_4 polynucleotide kinase and [$\gamma^{32}P$]ATP and the sequencing reactions were carried out with the use of *fmol* DNA Cycle Sequence System from Promega Corp. Two nucleotide sequences of 544 bp (SEQ ID No. 4) and 395 bp (SEQ ID No. 6), respectively, have been determined and shown as follows.

5'- GGGCC CTTCC TCGAG GCGGT GTCCC ACCTG CCGCC CTTCT TCGAT
TGCCT TGGGT CCCCA GTGTT TACTC CCATC AAGGC AGACA TAAGC GGCAA
CATCA CGAAA ATCAA AGCTG TGTAC GACAC CAACC CAGCC AAGTT CCGGA
CCCTG CAGAA CATCC TGGAG GTGGA GAAAG AAATG TATGG AGCAG AGTGG
CCCAA AGTAG GGGCC ACACT GGCGC TGATG TGGCT GAAAA GAGGC CTCGT
TCATC AGGTC TTCCT CCAGA GCATC TGCGA CGGGG AGCGG GACGA GAACC
ACCCC AACCT CATCC GTGTC AACGC CACCA AGGCC TACGA GATGG CCCTC
AAGAA GTACC ATGGC TGGAT CGTGC AGAAG ATCTT CAGGC AGCAC TGTAC
GCAGC ACCCT ATAAG TCTGA CTTCT GAAAG CGCTC TCCAA GGGGC AGAAT
GTTAC GGAGG AGGAG TGCTG GAGAA GATCC GCTCT TCTAG TCAAC TACAC
GGGAC CATCG ATGTC ATCTA CGAGA TGTAC ACCAG ATGAA CGCTG AGCT
(SEQ ID No. 4)

The corresponding coding sequence for SEQ ID No. 4 is

GPFLE AVSHL PPFFD CLGSP VFTPI KADIS GNITK IKAVY DTNPA KFRTL
QNILE VEKEM YGAEW PKVGA TLALM WLKRG LVHQV FLQSI CDGER DENHP
NLIRV NATKA YEMAL KKYHG WIVQK IFRQH CTQHP ISLTS ESALQ GAECY
GGGVL EKIRS SSQLH GTIDV IYEMY T (SEQ ID No. 5)

5'-AAATA GCTGG CATGT GCGAC GCTTG AGTCA GCTAC TAGAG CTAGC
AGGAG AATCG CTTGA CCGAG TGAGT TGCAG TGAGC TGAGA TCGCA CCATT
GCACT CCAGC CTGGG CAACC AGAGC GAAAC TCTGT CTCAA AAAAA AAAAA
AAAAG AGGTG GGTGG ATTAC TTGAG GTCAG GGTTT GAGAT CAGCC TGACC
AACAT GGTGA AACCC TATCT CTACT AAAAA TATAG AATTA GCCAG GCATG
GTAGC GCACG CCTGT AATCC CATCT TCTTG GGAGG CTGAG GCAGG AGAAT

CGCTA GAACC TGGAG GTGGA GGTTA CAGTA GCCGA GATCG CGCCA CTGCA TTCCA GCCTG GGCAA CAAAA GCGAA ACTCT GTCTC AAAAA AGAAA AAAAA (SEQ ID No. 6)

A deposit of this vector has been made with the American Type Culture Collection (Rockville, MD) on March 10, 1998, under the terms of the Budapest Treaty. ATCC Accession number _____ has been assigned to the deposit. The angiogenin receptor DNA fragment can be amplified and isolated from the deposited clone 8 by PCR technique with two oligonucleotide primers that match the sequence flanking the EcoR I insertion site of lambda gt11 genome. The sequence of these two primers are 5'-d(GGTGGCGACGACTCCTGGAGCCG)-3'and 5'-(TTGACACCAGACCAACTGGTAATG)-3', respectively.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Vallee, Bert L.
Hu, Guo-Fu

(ii) TITLE OF INVENTION: Angiogenin Receptor, Compositions and Methods Related Thereto

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Ala Ile Arg Ala Ser Asn Ile Glu Lys Pro Leu Gly Tyr Phe
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Gly Val Thr Val Leu Ile Val Asn Gln Gln Glu Phe Xaa Arg Ala
1 5 10 15

Leu Ala Ala Phe
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

His Trp Tyr Pro Asn Tyr Leu Gly Met
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 544 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGCCCTTCC TCGAGGCGGT GTCCCACCTG CCGCCCTTCT TCGATTGCCT TGGGTCCCCA
60

GTGTTTACTC CCATCAAGGC AGACATAAGC GGCAACATCA CGAAAATCAA AGCTGTGTAC
120

GACACCAACC CAGCCAAGTT CCGGACCCTG CAGAACATCC TGGAGGTGGA GAAAGAAATG
180

TATGGAGCAG AGTGGCCCAA AGTAGGGGCC ACACTGGCGC TGATGTGGCT GAAAAGAGGC
240

CTCGTTCATC AGGTCTTCCT CCAGAGCATC TGCGACGGGG AGCGGGACGA GAACCACCCC
300

AACCTCATCC GTGTCAACGC CACCAAGGCC TACGAGATGG CCCTCAAGAA GTACCATGGC
360

TGGATCGTGC AGAAGATCTT CAGGCAGCAC TGTACGCAGC ACCCTATAAG TCTGACTTCT
420

GAAAGCGCTC TCCAAGGGGC ACGATGTTAC GGAGGAGGAG TGCTGGAGAA GATCCGCTCT
480

TCTAGTCAAC TACACGGGAC CATCGATGTC ATCTACGAGA TGTACACCAG ATGAACGCTG
540

AGCT
544

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Pro Phe Leu Glu Ala Val Ser His Leu Pro Pro Phe Phe Asp Cys
1 5 10 15

Leu Gly Ser Pro Val Phe Thr Pro Ile Lys Ala Asp Ile Ser Gly Asn
20 25 30

Ile Thr Lys Ile Lys Ala Val Tyr Asp Thr Asn Pro Ala Lys Phe Arg
35 40 45

Thr Leu Gln Asn Ile Leu Glu Val Glu Lys Glu Met Tyr Gly Ala Glu
50 55 60

Trp Pro Lys Val Gly Ala Thr Leu Ala Leu Met Trp Leu Lys Arg Gly
65 70 75 80

Leu Val His Gln Val Phe Leu Gln Ser Ile Cys Asp Gly Glu Arg Asp

85 90 95

Glu Asn His Pro Asn Leu Ile Arg Val Asn Ala Thr Lys Ala Tyr Glu
100 105 110

Met Ala Leu Lys Lys Tyr His Gly Trp Ile Val Gln Lys Ile Phe Arg
115 120 125

Gln His Cys Thr Gln His Pro Ile Ser Leu Thr Ser Glu Ser Ala Leu
130 135 140

Gln Gly Ala Glu Cys Tyr Gly Gly Gly Val Leu Glu Lys Ile Arg Ser
145 150 155 160

Ser Ser Gln Leu His Gly Thr Ile Asp Val Ile Tyr Glu Met Tyr Thr
165 170 175

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 395 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAATAGCTGG CATGTGCGAC GCTTGAGTCA GCTACTAGAG CTAGCAGGAG AATCGCTTGA
60

CCGAGTGAGT TGCAGTGAGC TGAGATCGCA CCATTGCACT CCAGCCTGGG CAACCAGAGC
120

GAAACTCTGT CTCAAAAAAA AAAAAAAAAG AGGTGGGTGG ATTACTTGAG GTCAGGGTTT
180

GAGATCAGCC TGACCAACAT GGTGAAACCC TATCTCTACT AAAAATATAG AATTAGCCAG
240

GCATGGTAGC GCACGCCTGT AATCCC'TCT TCTTGC GAGG CTGAGGCAGG AGAATCGCTA
300

GAACCTGGAG GTGGAGGTTA CAGTAGCCGA GATCGCGCCA CTGCATTCCA GCCTGGGCAA
360

CAAAAGCGAA ACTCTGTCTC AAAAAAGAAA AAAAA
395

We Claim:

1. An isolated and/or recombinant polypeptide comprising a mammalian angiogenin receptor amino acid sequence.

2. An isolated and/or recombinant polypeptide comprising a human angiogenin receptor amino acid sequence.

3. The polypeptide of claim 1 or 2, wherein the angiogenin receptor sequence is sufficient for binding to angiogenin.

4. The polypeptide of any of claims 1-3, wherein the angiogenin receptor sequence is encoded by a nucleic acid which hybridizes to coding sequence of one or both of SEQ ID Nos: 4 and 6.

5. The polypeptide of any of claims 1-3, wherein the angiogenin receptor sequence is cross-reactive with an antibody which is selective for a peptide of SEQ ID Nos: 1, 2, 3 or 5.

6. The polypeptide of any of claims 1-3, wherein the angiogenin receptor sequence is encoded by a nucleic acid which hybridizes under stringent conditions with the nucleic acid sequence of ATCC deposit _____.

7. An isolated and/or recombinant polypeptide comprising an angiogenin receptor amino acid sequence identical or homologous to an amino acid sequence represented in SEQ ID No. 1, 2, 3 and/or 5.

8. The polypeptide of claim 7, wherein the angiogenin receptor sequence is sufficient for binding to angiogenin.

9. An isolated and/or recombinant angiogenin receptor polypeptide encoded by a nucleic acid which hybridizes under stringent conditions to a mammalian angiogenin receptor gene.

10. The polypeptide of any of claims 1-9, wherein angiogenin receptor sequence modulates proliferation of endothelial cells

11. The polypeptide of any of claims 10, wherein angiogenin receptor sequence modulates migration of endothelial cells

12. The polypeptide of any of claims 1-11, wherein angiogenin receptor sequence has a kinase activity.

13. The polypeptide of any of claims 1-12, wherein the polypeptide is soluble.

14. An isolated extracellular fragment of a mammalian angiogenin receptor, which fragment retains angiogenin binding activity.

15. The polypeptide of claims 1, 2, 7 or 14, which polypeptide is a fusion protein.

16. An isolated and/or recombinant antibody specifically reactive with an epitope of the mammalian angiogenin receptor polypeptide.

17. The antibody of claim 16, which is a monoclonal antibody.

18. An isolated nucleic acid comprising a coding sequence for a polypeptide of any of claims 1-15.

19. An isolated nucleic acid comprising a coding sequence for a mammalian angiogenin receptor polypeptide.

20. An isolated nucleic acid comprising a coding sequence which hybridizes under stringent conditions to the AR coding sequence of ATCC deposit _____.

21. The nucleic acid of claims 18, 19 or 20, further comprising a transcriptional regulatory sequence operably linked to the coding sequence so as to render the nucleic acid suitable for use as an expression vector.

22. An expression vector, capable of replicating in at least one of a prokaryotic cell and eukaryotic cell, comprising the nucleic acid of claim 21.

23. A host cell transfected with the expression vector of claim 22 and expressing the recombinant polypeptide.

24. A method of producing a recombinant angiogenin receptor polypeptide comprising culturing the cell of claim 23 in a cell culture medium to cause expression of an angiogenin receptor polypeptide encoded by the expression vector.

25. A transgenic animal having cells which harbor a transgene comprising the nucleic acid of claim 18, 19 or 20.

26. A transgenic animal in which angiogenin receptor stimulated signal transduction pathways are inhibited in one or more tissue of the animal by one of either expression of an antagonistic angiogenin receptor polypeptide or disruption of an angiogenin receptor gene.

27. A nucleic acid comprising a substantially purified oligonucleotide, the oligonucleotide containing a region of nucleotide sequence which hybridizes under

stringent conditions to at least 10 consecutive nucleotides of sense or antisense sequence of SEQ ID No. 4 or 6, or naturally occurring mutants thereof.

28. The nucleic acid of claim 27, which nucleic acid further comprises a label group attached thereto and able to be detected.

32. A test kit for detecting cells which contain an angiogenin receptor mRNA transcript, comprising a nucleic acid of claim 27 for measuring, in a sample of cells, a level of nucleic acid encoding an angiogenin receptor protein.

33. A test kit for detecting cells or tissue containing an angiogenin receptor protein, comprising an antibody specific for the angiogenin receptor protein for measuring, in a sample of cells, a level of the angiogenin receptor protein.

34. A method for modulating, in an animal, cell growth, differentiation or survival, comprising administering a therapeutically effective amount of an angiogenin receptor polypeptide.

35. The method of claim 34, comprising administering a nucleic acid construct encoding the angiogenin receptor polypeptide under conditions wherein the construct is incorporated and recombinantly expressed by the cells to be modulated or cells located proximate thereto.

36. The method of claim 34, wherein the angiogenin receptor protein is a soluble extracellular fragment of the receptor which antagonizes angiogenin.

37. A method for treating undesired angiogenesis in a human or animal comprising the step of administering to the human or animal with the undesired angiogenesis a composition comprising an effective amount of an inhibitor of angiogenin-dependent angiogenin receptor function.

38. The method of claim 37, wherein the inhibitor is a compound which competitively inhibits binding of angiogenin by the angiogenin receptor, an antisense construct which inhibits expression of an angiogenin receptor, or a angiogenin kinase inhibitor.

39. The method of claim 37, wherein the method is used in the treatment or prevention of undesired angiogenic process as may be associated with retinal neovascularization, choroidal neovascularization, diabetic retinopathy, macular degeneration, corneal neovascularization, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A, contact lens overwear, atopic keratitis, superior

limbic keratitis, pterygium keratitis sicca, sogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections other than leprosy, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi's sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, trauma, rheumatoid arthritis, systemic lupus, polyarteritis, Wegeners sarcoidosis, scleritis, Steven Johnson's disease, radial keratotomy, sickle cell anemia, sarcoid, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occulsion, carotid obstructive disease, chronic uveitis, chronic vitritis, Lyme disease, Eales disease, Bechets disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, post-laser complications abnormal proliferation of fibrovascular tissue, hemangiomas, Osler-Weber-Rendu, solid tumors, blood borne tumors, acquired immune deficiency syndrome, ocular neovascular disease, osteoarthritis, diseases caused by chronic inflammation, Crohn's disease, ulceritive colitis, tumors of rhabdomyosarcoma, tumors of retinoblastoma, tumors of Ewing sarcoma, tumors of neuroblastoma, tumors of osteosarcoma, leukemia, psoriasis, atherosclerosis, or pemphigoid.

40. A recombinant transfection system, comprising

(i) a gene construct encoding an angiogenin receptor polypeptide and operably linked to a transcriptional regulatory sequence for causing expression of the angiogenin receptor polypeptide in eukaryotic cells, and

(ii) a gene delivery composition for delivering the gene construct to a cell and causing the cell to be transfected with the gene construct.

41. The recombinant transfection system of claim 40, wherein the gene delivery composition is selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent.

42. A method of determining if a subject is at risk for a disorder characterized by unwanted cell proliferation, differentiation or death, comprising detecting, in a tissue of the subject, the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a gene encoding an angiogenin receptor protein; and (ii) the mis-expression of the gene.

43. The method of claim 42, wherein detecting the genetic lesion comprises ascertaining the existence of at least one of

i. a deletion of one or more nucleotides from the gene,

ii. an addition of one or more nucleotides to the gene,
iii. an substitution of one or more nucleotides of the gene,
iv. a gross chromosomal rearrangement of the gene,
v. aberrant methylation of the gene,
vi. a gross alteration in the level of a messenger RNA transcript of the gene,
vii. the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene, and
viii. a non-wild type level of the protein.

44. The method of claim 42, wherein detecting the genetic lesion comprises
i. providing a nucleic acid comprising an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence of a mammalian angiogenin receptor gene, such as SEQ ID No. 4 or 6, or naturally occurring mutants thereof or 5' or 3' flanking sequences naturally associated with the gene;
ii. exposing the nucleic acid to nucleic acid of the tissue; and
iii. detecting, by hybridization of the nucleic acid to the nucleic acid, the presence or absence of the genetic lesion.

45. The method of claim 43, wherein detection of the genetic lesion comprises detecting the presence or absence of an angiogenin receptor protein in cells of a tissue sample and/or as soluble proteins in bodily fluid.

46. A method of detecting the presence of an angiogenin receptor ligand on cells present in a biological sample, comprising contacting the cells with a labeled angiogenin receptor polypeptide and under conditions where the angiogenin receptor polypeptide can specifically bind to cognate ligand, and detecting presence of the angiogenin receptor polypeptide bound to the cells.

47. An assay for screening test compounds that modulate the bioactivity of an angiogenin receptor comprising:
i. combining a test compound, an angiogenin receptor polypeptide, and a target compound selected from the group consisting of an angiogenin receptor ligand, a signal transduction protein which binds to the angiogenin receptor polypeptide, or a substrate of a kinase activity of the angiogenin receptor polypeptide; and

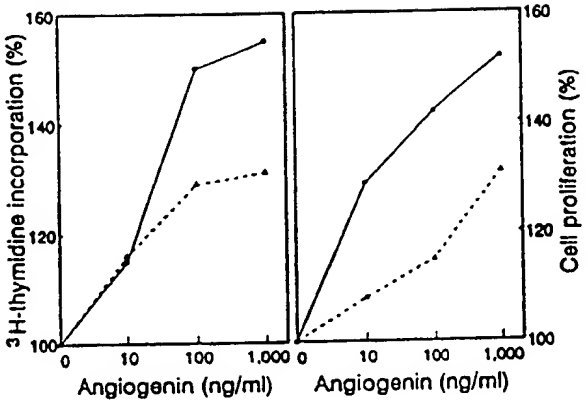
ii. detecting the interaction of the target compound and the angiogenin receptor polypeptide,

wherein a change in the interaction of the target compound and the angiogenin receptor polypeptide in the presence of the test compound is indicative of a potential ability to modulate the bioactivity of the angiogenin receptor.

48. The assay of claim 47, wherein the angiogenin receptor polypeptide is a soluble polypeptide.

Figure 1

FIG 1A FIG 1B



BNSDOCID <WO_ 9840487A1_I >

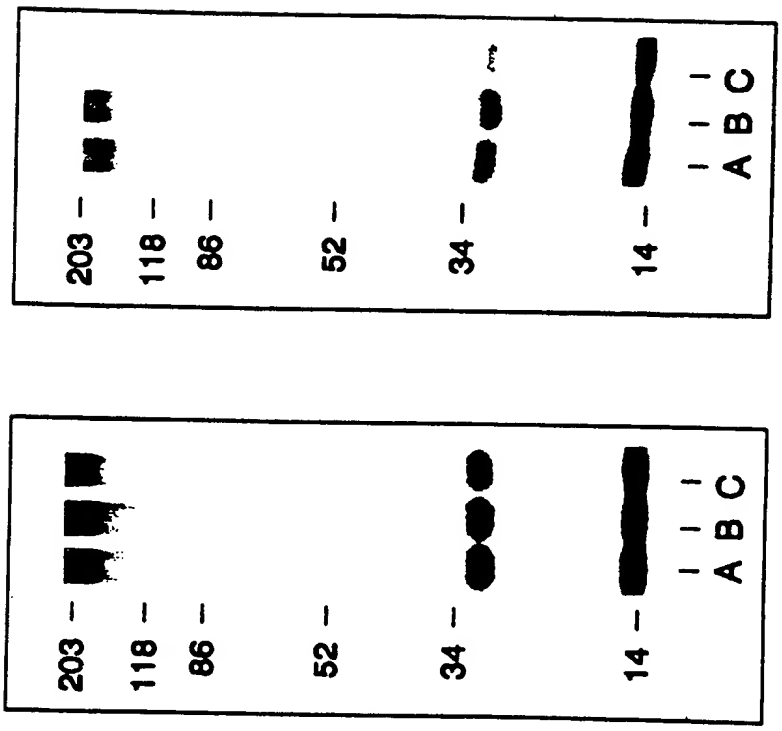


FIG 2

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Figure 3

FIG 3A

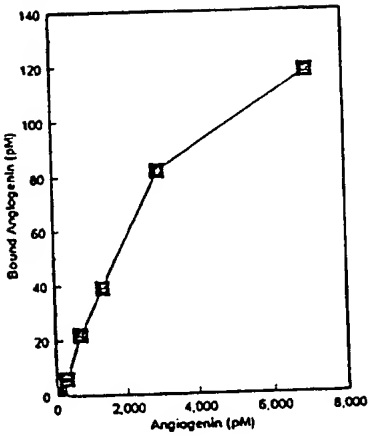
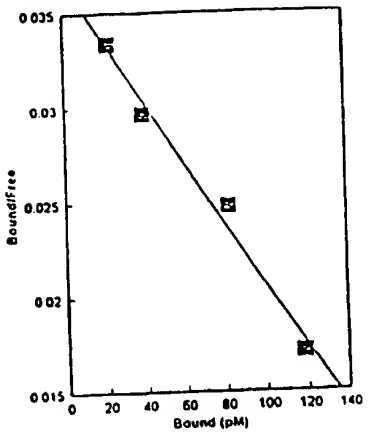


FIG 3B



BNSDOCID <WO 9840487A1 I >

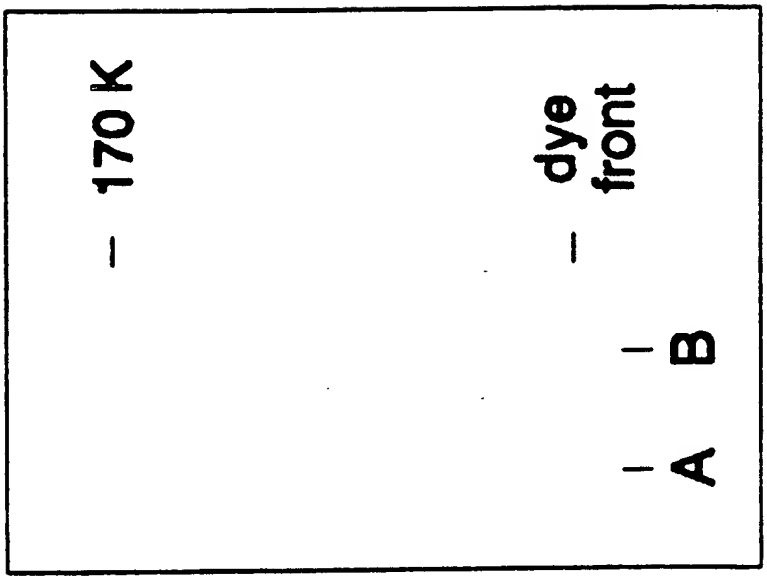


FIG 4

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/05176

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/71 A01K67/027 C07K16/28 C12N15/62
C12N15/11 C12Q1/68 A61K38/17 G01N33/68 C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ˚	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABE, A. ET AL.: "Primary structure of glycolipid transfer protein from pig brain." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 17, 15 June 1990, pages 9634-7, XP002072675 see figure 1 ---	1,2,4-6, 13
X	CHAMOUX, M. ET AL.: "Characterization of angiogenin receptors on bovine brain capillary endothelial cells." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 176, no. 2, 30 April 1991, pages 833-9, XP002072676 cited in the application see the whole document --- -/--	1,3,10, 46-48

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

˚ Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LEVY-LAHAD, E. ET AL.: "Genomic structure and expression of STM2, the chromosome familial Alzheier disease gene." GENOMICS, vol. 34, 1996, pages 198-204, XP002072677 see the whole document -& DATABASE EMBL -EMHUM2 Entry 50871, aCC.nO. U50871, 8 July 1996 LEVY-LAHAD, E. ET AL.: "HUMAN FAMILIAL aLZHEIMER'S DISEASE (stm2) GENE, COMPLETE CDS." XP002072776 see the whole document	18,20,27
X	DATABASE EMBL - EMEST10 Entry HSU51148, Acc.No. U51148, 1 January 1997 LIN, B. ET AL.: "Human clone 17N11 mRNA sequence." XP002072679 see the whole document	18,20,27
X	DATABASE EMBL - EMEST12 Entry MM4322, Acc.No. W09432, 29 April 1996 MARRA, M. ET AL.: "ma08h05.r1 Soares mouse p3NMF19.5 Mus musculus cDNA clone 303993 5' similar to SW:GLTP_PIG P17403 GLYCOLIPID TRANSFER PROTEIN ;." XP002072680 see the whole document	18,20,27
P,X	HU, G-F. ET AL.: "A putative angiogenin receptor in angiogenin-responsive human endothelial cells." PROC.NATL.ACAD.SCI.USA, vol. 94, 18 March 1997, pages 2204-9, XP002072678 see the whole document	1-5, 7-11,13, 46-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/05176

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see further information sheet enclosed

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 34-39 are directed to a method of treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compounds/composition.

Although claims 42-44 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compounds/composition.